EVALUATIONS OF DIAMINOALKANE MEDIATED IMMOBILIZED HEPARIN

by

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A dissertation submitted to the faculty of
The University of Utah
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Pharmaceutics

The University of Utah

December 1981

THE UNIVERSITY OF UTAH GRADUATE SCHOOL

SUPERVISORY COMMITTEE APPROVAL

of a dissertation submitted by

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This dissertation has been read by each member of the following supervisory committee and by majority vote has been found to be satisfactory.

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FINAL READING APPROVAL

To the Graduate Council of The University of Utah:

I have read the dissertation of Charles D. Ebert in its final form and have found that (1) its format, citations, and bibliographic style are consistent and acceptable; (2) its illustrative materials including figures, tables, and charts are in place; and (3) the final manuscript is satisfactory to the Supervisory Committee and is ready for submission to the Graduate School.

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ABSTRACT

This dissertation entails the immobilization of heparin to polymer substrates and the characterization of the resultant heparin immobilized surfaces. To prevent crosslinking, all derivatization and immobilization reactions were conducted with N-acetylated heparin.

Preliminary heparin derivatization studies demonstrated that carboxylic groups could be utilized in the covalent immobilization reactions if the number of participating carboxylic groups was minimized.

It was found that the anticoagulant activity of the immobilized heparin increased with increasing spacer group length separating the immobilized heparin from the polymer surface.

X-ray photoelectron spectroscopy revealed that the immobilized heparin was not associated with the surfaces of adsorbed protein layers following contact with protein solutions or plasma.

These results suggested that the immobilized heparin was masked from platelets and unable to directly interact with platelets as is the case with soluble heparin. This hypothesis was substantiated by platelet retention and degranulation studies.

CONTENTS

ACKNOWLEDGMENTS

I wish to express my deepest appreciation to Dr. S.W. Kim for his support and guidance of this work and to Dr. J.D. Andrade for the use of the XPS spectrometer system and for the enlightening discussions. I also would like to thank all the members of my Supervisory Committee for their invaluable suggestions.

This work was supported by NIH Grant HL 20251.

CHAPTER 1

INTRODUCTION

1.1 Commercial Preparation

Heparin is the most commonly used anticoagulant. Since first becoming commercially available in the late 1930's (1), more than 6 metric tons are now annually produced primarily from bovine lung and bovine and porcine intestinal mucosa (2). Heparin is produced by mast cells as a macromolecular complex composed of linear polyanionic carbohydrate chains covalently linked to a protein core, the total molecular weight of the macromolecular complex ranging from 750,000 to 1,000,000 (3). The separation and purification of heparin is complicated by the presence in the same source tissues of compounds possessing similar chemical and physical properties, but with no anticoagulant activity (4). These compounds, including hyaluronic acid, chrondroitin sulfates A, B, C and D, and keratin sulfate, also exist in the native state as covalently conjugated proteo-glycuronans and heparin composes less than 1% of that mixture (5). The protein moiety is removed either by alkaline or enzymatic hydrolytic cleavage to yield a glycosaminoglycuronan mixture from which commercial heparin is separated. Although it has been generally accepted that mast cells were the only certain source of the native heparin protein complex, recent studies have shown the existence of heparin-protein complexes in

liver and muscle tissues (6) and evidence for the existence of a placental heparin-protein complex (7,8). However, none of these sources are of commercial importance.

1.2 Chemical Structure

Commercial heparin preparations exist as a population of sulfoglucosamine and hexuronic acid units linked by 1-4 glycosidic bonds to form linear anionic carbohydrate molecules ranging in molecular wieght from less than 10,000 to greater than 20,000 (9, 10). Degradation of commercial heparin by enzymes of *Flavorbacterium heparinum* has revealed that over 90% of the total heparin preparation exists as a hexasaccharide containing 2,6-disulfoglucosamine, 2-sulfoiduronic acid and glucuronic acid in 3:2:1 proportions respectively (2). Biosynthesis studies have shown that single sugar units in the heparin hexasaccharide chain can vary; for example, both sulfamino, acetylamino and free amino glucosamine may occur and iduronic and glucuronic acid may occur in both sulfated and unsulfated forms (11). Changes in any of the above structural parameters correlated at best with limited success to the anticoagulant activity of heparin.

Individual sugar unit variations, combined with the large molecular weight variations among heparin molecules within a commercial preparation, leads to a high degree of polydispersity. The high polydispersity of heparin, in size and chemical structure, is born out by findings that after electrophoresis and staining with toluidine blue, a basic dye that complexes acidic components in-

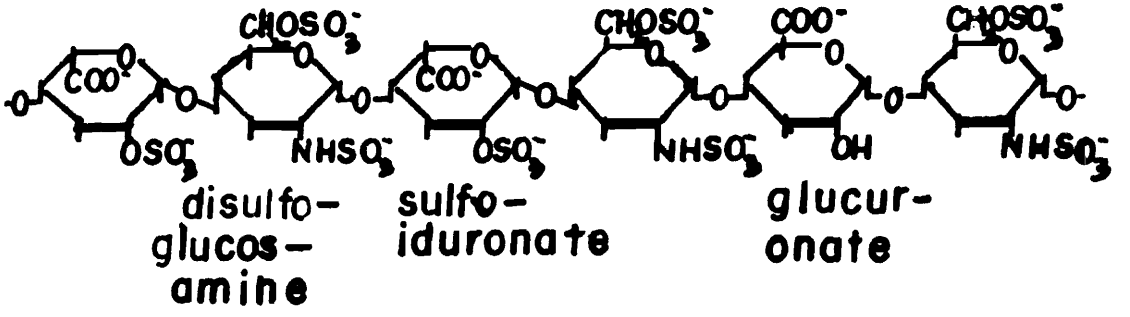
cluding heparin, at least 21 types of heparin molecules can be isolated with the same charge and the molecular weight of those components ranges from 3,000 to 37,500 (3).

In view of the ill-defined chemical structure of heparin, numerous investigators have considered the possibility that the anticoagulant activity of heparin preparations is associated with only a small fraction of the total heparin molecules present in a given preparation, the remaining molecules representing gross impurities, an analogous situation to the separation and purification of commercial heparin from the mixture at glycosaminoglycuronans. In an attempt to isolate an homogenous heparin species which could be correlated to the anticoagulant activity of heparin preparations, a large excess of antithrombin III (ATIII), the relevance of ATIII to heparin anticoagulant activity is described below, was added to a commercial heparin preparation. Subsequent sucrose gradient centrifugation revealed two distinct populations of heparin, one population that bound ATIII and a second population did not bind ATIII (12). The ATIII binding heparin population represented less than one-third of the heparin preparation yet it possessed over 85% of the total anticoagulant activity of the starting material. These active heparin species also proved to be highly polydispersed, ranging in molecular weight from $\sim$ 7,000 to $\sim$ 20,000. Detailed chemical-structural analysis of molecular weight fractions obtained from active (binds ATIII) and inactive (does not bind ATIII) heparin populations revealed the existence at a unique tetrasaccharide sequence present in the

active heparin population (13,14). The tetrasaccharide sequence, L-iduronic acid → N-acetylate-D-glucosamine 6-sulfate → D-glucuronic acid → N-sulfated-D-glucosamine 6-sulfate, was found in only 2.6% of the molecules present in the inactive heparin population while every molecule in the active population possess at least one tetrasaccharide sequence (13). Paradoxically, the L-iduronic acid is not sulfated in the ATIII binding tetrasaccharide sequence, the vast majority of those sugar units being present as 2-sulfoiduronic acid, and the relative abundance of sulfates in the tetrasaccharide sequence is substantially lower than for the bulk heparin preparation; yet it has long been established that minimal heparin desulfination leads to a rapid loss in anticoagulant activity (15).

The correlation between the relative abundance of the unique tetrasaccharide sequence and the anticoagulant activity strongly suggests that this sequence represents the ATIII binding site. This contention is further supported by findings that the tetrasaccharide sequence is protected from the enzymatic degradative action of bacterial heparinase when the active heparin molecules are in contact with ATIII (14).

A composite chemical structure for heparin begins to emerge, heparin being composed primarily of the previously mentioned hexasaccharide units, which can vary considerably in individual sugar unit structure, with sporadically located ATIII binding tetrasaccharide units. The hexasacchride sequence and the ATIII binding tetrasacchride sequence are shown in Figure 1. Any combina-



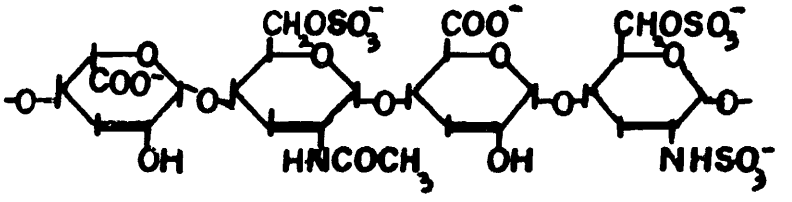


Figure 1. Heparin tetrasacchride and antithrombin III binding site shown above and below respectively.

tion of these two sequences appears possible; however, only molecules with at least one ATIII binding sequence possess anticoagulant activity.

Other chemical groups are also observed in heparin structure only to a much smaller extent. It has been shown that commercial heparin also contains between 0.3% and 2.3%, depending upon the source, amino acids. Of those amino acids, glycine is the most prominent, followed by cysteic acid, alanine, aspartic acid and serine in that order of abundance (12). These amino acids can be covalently linked to heparin, in the specific case of aspartic acid being linked via an N-glycosyl bond between the carboxylic groups on aspartic acid to amino groups of hexosamines, or they may simply be ionically bound and represent impurities from the native heparin protein moiety.

In an attempt to correlate heparin structural parameters to its anticoagulant activity, numerous investigators have prepared synthetic heparinoids, primarily linear sulfated carbohydrates (16,17). Although these materials mimic heparin to limited degrees, they do not appear to act with coagulation factors to the same extent as heparin, leading to reduced potency relative to heparin. A novel approach to synthetic heparinoids has recently been reported, where the double bonds in cis-polyisoprene are converted to adjacent N-sulfates and carboxylic functional groups to provide an improved structural similarity to heparin (18). These materials have improved anticoagulant potency; however, they still do not interact with coagulation factors in an identical manner as

heparin, as evidenced by differences in activities with different coagulation assays.

Heparin has been shown to complex with different coagulation factors, such as thrombin, ATIII, factor Xa, platelet factors and numerous enzymes as discussed below. It is apparent that heparin must possess multiple structural domains for interaction with these various factors. Isolation of the ATIII binding site of heparin does not, by itself, describe the structural requirements for the in vivo anticoagulant effect of heparin. Carbohydrate sequences residing outside of the primary ATIII binding tetrasaccharide sequence may play an important role in the anticoagulant activity of heparin. Furthermore, spatial orientations of these structural domains must also play an essential role in the anticoagulant activity. An example of these conformational requirements for the structural domains is provided in the ATIII binding tetrasaccharide. As previously mentioned, the iduronic acid in the tetrasaccharide is not sulfated while the majority of iduronic acid residues of heparin are sulfated. Sulfoiduronic acid is reported to possess only one stable conformational state while iduronic acid can assume three different conformations (19).

To better understand the structural requirements correlating to the anticoagulant activity, the dynamic conformational structures must also be considered. Undoubtedly, future work will reveal more information with these regards.

1.3 Heparin Pharmacology

Heparin exerts its anticoagulant effect by preventing the conversion of fibrinogen to fibrin via the proteolytic enzyme thrombin. The formation of thrombin occurs primarily via two pathways, intrinsic and extrinsic, in which activation of zymogen plasma coagulation factors initiates activation of subsequent factors in the progression of each pathway. This cascade of sequential coagulation factor activations is presented in Figures 2 and 3.

Activation of the intrinsic pathway is initiated by conversion of factor XII (Hageman factor) to the proteolytic active species factor XIIa. This initial activation step can occur through a variety of stimulants, the most common being through interaction with exposed collagen brought about following injury to the endothelial cell lining of the vascular intima (20). Other stimulants are also known to activate factor XII, including adsorption onto negatively charged foreign surfaces (20). Factor XII interactions with negatively charged surfaces can result in the formation of two active species (21), one which remains bound to the surface and can activate subsequent factors, factor XI as described below, only if they too are surface associated, and the second being a soluble factor XIIa fragment which will not activate factor XI. This factor XIIa fragment will, however, induce conversion of prekallikrein to kallikrein, a proteolytic enzyme which can cleave high molecular weight kininogens into bradykinin, a potent peripheral vasodilator. Factor XIIa in turn converts

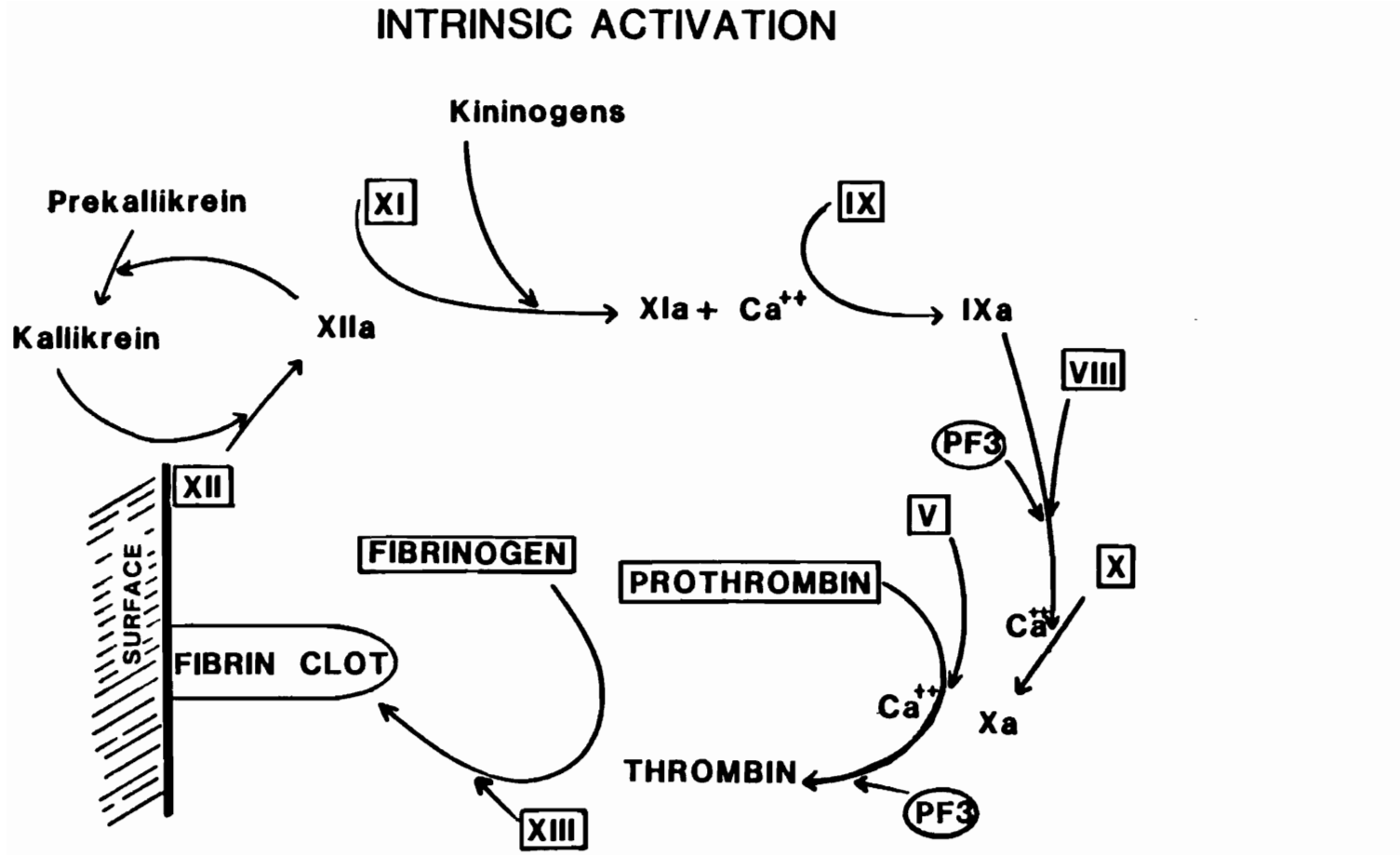


Figure 2. Intrinsic Coagulation Pathway

EXTRINSIC ACTIVATION

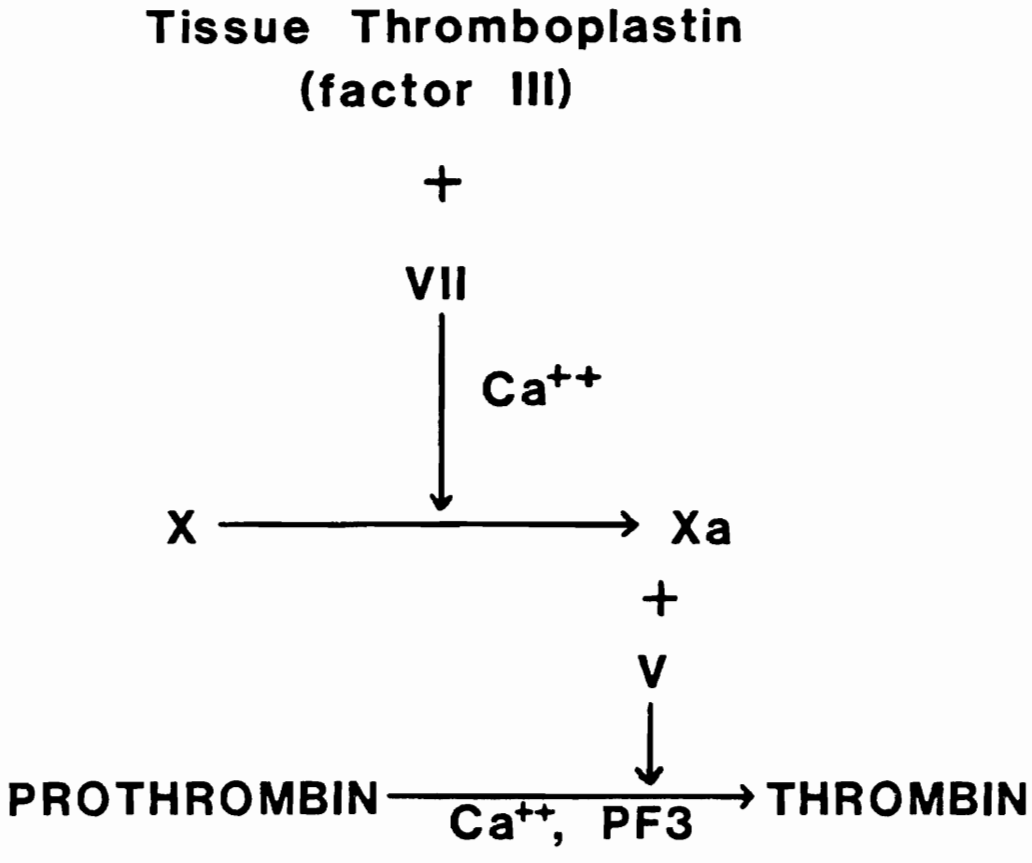


Figure 3. Extrinsic Coagulation Pathway

factor XI to factor XIa which subsequently converts factor IX to factor IXa. Factor IXa combines with lipids (platelets) and a co-factor, factor VIIIa, to form a complex that converts factor X to factor Xa (20,22). Once factor Xa has been produced, it combines with phosopholipid surfaces (platelet membranes) and factor Va to form a complex that insures rapid conversion of prothrombin to thrombin. Although the formation of the factor Xa:factor Va: phospholipid surface complex is not absolutely necessary for conversion of prothrombin to thrombin by factor Xa, prothrombin activation kinetics are much more rapid in the presence of the phospholipid surface and factor VIIIa cofactors (22). Thrombin in turn converts fibrinogen to fibrin monomer which is then cross-linked into a fibrin network by factor XIII.

Activation of the extrinsic pathway is initiated by tissue injury with resultant release of a protein-lipid tissue factor, which is not yet completely identified, that combines with factor VII (23) to form a complex that converts factor X to factor Xa and conversion of prothrombin to thrombin occurs through the pathway previously described.

Platelets can also initiate activation of prothrombin by release of platelet factor 3, a substance believed to be associated with the platelet membrane (24).

Activation of coagulation mechanisms is modulated by the presence of several natural inhibitors, antithrombins (AT), in blood. These antithrombins serve a vital function; for in their absence, once prothrombin is converted to thrombin, 10 ml of blood would

contain a sufficient quantity of thrombin to clot all of the fibrinogen in the body (25).

Six different antithrombins have been described, however, only three are known to play a significant role in the modulation of coagulation: ATI, ATII and ATIII. Of these three, ATIII is the major thrombin inhibitor in plasma and the plasma cofactor through which heparin exerts its anticoagulant effect (26). Antithrombin III is present in normal plasma at an average concentration of ∿ 150 μg/ml. When ATIII is added to thrombin, a slow decline in thrombin activity is observed. Using purified protein it has been shown that thrombin combines with ATIII to form a 1:1 stoichiometric complex (26). However, when heparin is added to a thrombin:ATIII mixture, thrombin neutralization is essentially instantaneous and the stoichiometry of the thrombin:ATIII complex is unchanged. The formation of the thrombin:ATIII complex is critically dependent upon the active serine center of thrombin. When this amino acid is blocked, all interactions between thrombin and antithrombin III are eliminated. Thrombin cleaves arginine-glycine bonds in fibrinogen to form fibrin. Suspecting that thrombin binds to ATIII by a similar interaction, arginine on ATIII was blocked and it was found that thrombin could no longer complex with that ATIII (26). Similarily, in view of the high anionic character of heparin, it is likely that heparin would interact with positively charged groups on proteins such as lysine residues. Chemical blocking of these residues on ATIII produced an ATIII which interacted normally with thrombin, resulting in a

slow decline in thrombin activity. However, this blocked ATIII does not bind heparin and when heparin was added to a mixture of thrombin and lysine blocked ATIII, inactivation of thrombin was indistinguishable from that without heparin (26). These findings have lead to the generally accepted hypothesis that heparin prevents fibrinogen conversion to fibrin by first binding to ATIII to form a heparin:ATIII complex in which the thrombin binding site on the ATIII component of the complex is somehow more accessible to the active serine site on the protease enzyme. The heparin:ATIII complex then binds and neutralizes thrombin. This hypothesis is further supported by the fact heparin binding does induce conformational changes in ATIII (27).

If the hypothesis that an arginine binding site on ATIII interacts with active serine sites is correct, then ATIII should bind and neutralize every active serine protease in the intrinsic coagulating pathway (factors XIIa, IXa, XIa, and Xa) and heparin should greatly accelerate these reactions. This was found to be the case for factor XIIa (28), factor IXa (29) and factor XIa (30). However, the affects of heparin on factor Xa inhibition have been observed to be quite different than with thrombin (31). Although ATIII does slowly inhibit factor Xa activity, substantially less heparin is required to inhibit factor Xa in the presence of ATIII than to inhibit thrombin, and a linear relationship exists between the log of factor Xa activity and incubation time. With thrombin in the presence of ATIII, heparin inhibits the proteolytic enzyme activity essentially instantaneously, suggesting

that heparin interacts via a different mechanism with factor Xa. The anticoagulant activities of different heparin molecular weight fractions supports the contention that heparin interacts via a different mechanism with factor Xa. Low molecular weight heparin has little effect on thrombin activity and a high antifactor Xa activity; whereas, high molecular weight heparin shows an opposite relationship (32). More recent studies have shown that ATIII possesses multiple heparin binding sites (33,34). Low molecular weight heparin can associate with ATIII to provide a 2:1 stoichiometric complex while high molecular weight heparin forms a 1:1 stoichiometric complex with ATIII (33), suggesting a cooperative heparin binding model where two heparin binding sites on ATIII are in close proximity to each other.

An increase in the rate of thrombin neutralization can be demonstrated at heparin concentrations as low as 0.02 units/ml (35). At this concentration, ATIII molecules outnumber heparin molecules by more than 100 to 1, indicating that heparin acts in a catalytic fashion with thrombin and ATIII. This is substantiated by other findings. It has been proposed that heparin can be released for further ATIII binding after the thrombin:ATIII complex has been formed (36), and it has been demonstrated that heparin weakly binds to ATIII after formation of the thrombin: ATIII complex as compared with the strong binding associated with noncomplexed ATIII (37). These results have been confirmed by others (38) where it was demonstrated that binding of thrombin to the heparin:ATIII complex results in the discharge of heparin, the

thrombin:ATIII complexes having little ability to bind heparin, which can then bind noncomplexed ATIII with unchanged affinity.

More recent studies have shown that heparin contains both ATIII and thrombin binding sites (39,40,41) suggesting that thrombin is neutralized by binding to the heparin component of the heparin:ATIII complex; however, kinetic studies would indicate that this interaction is responsible for less than 2% of the thrombin neutralizing effect of the heparin:ATIII complex (42).

Currently, three models exists for heparin interaction with ATIII and thrombin. The widely accepted model has been that heparin first binds to ATIII, thereby potentiating binding of ATIII to thrombin. Heparin is then released from the ATIII:thrombin complex for eventual binding to other free ATIII molecules. In the second model heparin also first binds to ATIII and thrombin in turn binds to the heparin component of the heparin:ATIII complex; however, this model cannot explan the catalytic role of heparin. The third model proposes the existence of a heparin:ATIII:thrombin complex where both ATIII and thrombin are initially bound to the heparin (40). Other studies have shown that heparin can prevent activation of factor X in the presence of factor VIIIa, Ca^{+2}, and phospholipid surfaces, in the absence of ATIII (43); casting some doubt on the validity of the heparin:ATIII interactions being the primary mechanism for the anticoagulant effect of heparin. It should be noted, however, that when heparin is administered to normal blood for prophylactic control of coagulation, very few activated proteolytic coagulation factors exist relative to the

great abundance of ATIII and one would expect the heparin to bind to free ATIII. Whatever mechanisms or combinations of mechanisms heparin does exert anticoagulant effects is the subject of great controversy.

Heparin exerts other pharmacological functions, some indirectly affecting its anticoagulant activity while others are totally removed from intrinsic coagulation interactions. Heparin is known to adversely affect platelet functions (44). Heparin has been shown to independently induce platelet aggregation and to potentiate platelet aggregation and release reactions caused by exogenous agents (45); high molecular weight heparin fraction being more deleterious to platelet functions than low molecular weight fractions (45). Once aggregated, platelets can release substances which adversely effect hemostasis. Agents such as platelet factor 3 (PF3), and platelet factor 4 (PF4) can be released from platelets which can activate thrombin, in the case of PF3 (24), or bind to heparin and neutralize its anticoagulant activity in the case of PF4 (46). It has been shown that PF4 binds to different sites on the heparin molecule than the ATIII binding site (47), further demonstrating the complexity of multiple domains on the heparin molecule.

Heparin shows many other pharmacological functions and has been reported to affect the activity of more than 50 enzymes (2). Heparin can trigger the release of lipoprotein lipases from endothelium, adipose tissues and liver tissues (48) with consequent fatty acid level increases in plasma. Heparin initiates release

of histaminase from the intestinal mast cells (49), inhibits secretion at aldosterone (50) with resultant lower adrenal function, and triggers the release of monosomes and polysomes from rough endoplasmic reticulum (51).

In view of the complicated pharmacological effects associated with heparin and the danger of internal hemorrhaging from prolonged clotting times, an especially critical aspect to patients with hemophilic disorders and/or impaired hematopoietic function such as kidney dialysis patients, conventional heparin systemic administration can pose a high risk to the great number of individuals who routinely undergo treatment with blood contacting biomedical devices. To circumvent many of these undesired side reactions, many investigators have endeavored to incorporate heparin, and other anticoagulants, with the polymeric materials composing the blood contacting surfaces of such devices. The incorporation of heparin with biomedical polymeric materials composing blood contacting surfaces can be divided into two catagories: materials providing a controlled release of heparin at the blood polymer interface, either by an ion exchange mechanism or by diffusion through the polymer, and materials with surface immobilized heparin. In view of findings that heparin binds to vascular endothelium (2,52) to provide an increased negative surface charge (2), and a high interfacial concentration of heparin at the blood/vascular intima interface, this approach cannot only reduce side effects associated with systemic administration but also closely mimics the physiological behavior of heparin in vivo. A litera-

ture review of pertinent studies on heparin modification of biomedical materials follows.

1.4 Controlled Release Materials

1.4.1 Ionically Bound Heparin Polymers

The pioneering work in heparin controlled release materials was accidentally initiated by Gott, et al. (53). At that time, those investigators were evaluating numerous materials for thrombo-resistance by venous implantation and colloidal graphite gave the best results. These investigators first thought the thrombo-resistance to be due to first; the extreme smoothness on a microscopic level imparted to surfaces following graphite coating, and second; the chemically inert nature of carbon atoms composing the colloidal graphite. These materials were being sterilized by soaking in a Zepharin$^{(R)}$ (benzalkonium sulfate) solution. The surgeons then routinely soaked all implantable materials in a sterile heparin solution immediately prior to implantation. During the sterilization procedure, the hydrocarbon portion of the cationic surfactant was adsorbing to and penetrating into the graphite surface while the ionic portion was still associating with the aqueous phase. When these materials were placed in the heparin solution, heparin ionically exchanged with the sulfate anions to provide an ionically bound heparinized surface. When the heparinized materials were implanted, the heparin was slowly released from the graphite surface via an ion exchange mechanism

with anions of the circulating blood, thus providing a high interfacial concentration of heparin at the blood/polymer interface. Further studies showed that these graphite-benzalkonium-heparin (GBH) surfaces retained significant quantities of heparin even after 3 months implantation in the venous system (54).

A major disadvantage of the GBH surfaces is that the graphite can only be coated to rigid materials, any flexing would result in a desruption in the integrity of the graphite coating with possible "flaking-off" of the GBH coating. To circumvent this problem, Leininger, et al. (55) endeavored to chemically modify numerous polymer surfaces by forming permanent surface associated quaternary ammonium groups, eliminating the need for prior adsorption of a cationic surfactant onto a hydrophobic surface such as graphite. Depending upon the polymer three surface treatments were used: (1) chloromethylation of styrene followed by quaternization with dimethyl aniline; (2) radiation grafting of vinyl pyridine to numerous polymers followed by quaternization with methyl iodine or benzylchloride; and (3) incorporation of quaternizable monomers such as vinyl pyridine into copolymers. After quaternization the surfaces were placed in a heparin solution and heparin was ionically bound to the ammonium groups. Following contact with fibrinogen, γ-globulin and albumin solutions, Zeta potential measurements indicated that the surfaces were progressively becoming less negatively charged, which the author attributed to plasma protein adsorption. From these studies the authors attributed the nonthrombogenicity of the heparinized surfaces to alterations in

the plasma protein adsorption properties of the heparinized materials rather than to heparin release.

In an attempt to provide heparinized cellulose membranes that could be utilized in kidney dialysis applications, Merrill, et al. (56) ionically bound heparin to cellulose membranes via an ethylene imine intermediate. Various procedures were used to couple ethylene imine to the hydroxyl groups on cellulose. Of those procedures, pretreatment with ethylene oxide vapor, to convert secondary cellulose hydroxyl groups into primarily hydroxyl groups, followed by reacting ethylene imine in toluene produced aminated surfaces which could then ionically bind heparin. Ethylene imine reactions in aqueous solutions resulted in bulk amination of the cellulose, presumably due to increased swelling in water as opposed to toluene, which would ionically bind less heparin per unit surface area. However, surfaces prepared by reacting ethylene imine in 0.4 N HCl produced the greatest prolongations of whole blood clotting times. The authors attributed this finding to decreased cellulose crystallinity due to HCl dissolution of crystalline regions. Rather than attributing increased in vitro clotting times to desorption of heparin, having determined that the ionically bound heparin was not washed off in the time limits involved in the in vitro clotting time tests, the authors attributed the increase clotting times to the adsorption of coagulation factors. Evidence to this hypothesis was provided by plasma adsorption studies where it was found that heparinized cellulose ethylene imine surfaces adsorbed substantially greater

quantities of plasma proteins than either cellulose or ethylene imine treated cellulose surfaces. Platelet interaction studies by Salzman, et al. (57) on the cellulose:ethylene imine:heparin surfaces demonstrated high degrees of platelet adhesion *in vitro* and with sufficiently high surface areas, significant thrombocytopenia *in vivo* which could be reduced by pretreatment with albumin. Prior adsorption of albumin is known to produce passive surfaces with respect to platelet interactions (58). The authors concluded that adverse platelet interactions were due to alternations in plasma protein adsorption characteristics of the heparinized surfaces, possible resulting in increased fibrinogen adsorption, a protein known to increase platelet adhesion to pretreated surfaces (59).

Hersh, et al. (60) found that aminoorganosilane compounds could covalently bind to silica on glass surfaces previously cleaned with nitric acid. When γ-aminopropyltriethoxysilane (APTES) was refluxed in toluene with cleaned glass, a stable surface aminosilane was formed that could ionically bind heparin. This ionically bound heparin also appeared stably affixed to the surface and could resist fluid shear stresses as high as 10^4 dynes/cm^2 at 30^{o}C for over 300 hours. *In vitro* whole blood clotting times demonstrated a 10-fold increase in clotting times for the heparinized glass as opposed to untreated glass.

Realizing the applicability of the APTES surface treatment with silica filled silicone rubbers, a material widely used in artificial organs, Merker, et al. (61) similarly prepared hepari-

nized silicone rubber.

Grode, et al. (62) reported a simple procedure for ionically bonding heparin to silicone rubber. The silicone rubber was swollen in a 1:1 mixture of toluene and petroleum ether containing between 1-5% tridodecylmethylammonium chloride (TDMAC). The hydrocarbon portions of the quarternary amine adsorb onto and penetrates into the swollen silicone rubber, which was then vacuum dried. The TDMAC treated silicone rubber is then placed in a heparin solution and heparin was ionically bound to the quaternary amine. The same TDMAC treatment could be applied to numerous biomedical materials including, polyurethane, polypropylene, Mylar(R), polycarbonate, polyvinyl chloride and glass. The advantage of this procedure is that numerous materials could be heparinized without elaborate surface modification, the procedure is simple and all of these materials produced prolong clotting times. The disadvantages are: (L) TDMAC surfaces are unstable in plasma and TDMAC is released from the material and, (2) following release of heparin, TDMAC is directly exposed to the blood. These TDMAC surfaces in turn can adhere platelets.

Yen and Rembaum (63) reported the preparation of an ionically bound heparin material where a quaternizable monomer is incorporated into the copolymer chain via a commerically available polyether with terminal isocyanates. Subsequent quaternization with concentrated HCl resulted in the formation of cationic ammonium groups that could ionically bind between 7 to 16% by weight heparin, depending upon the abundance of the quaternizable monomer in

the copolymerization reaction. Blood could be stored in tubings coated with the heparinized elastomer for over 24 hours without clotting.

Tanzawa, et al. (64) described the preparation of a new hydrophilic heparinized polymer containing permanent quaternary ammonium groups in the copolymer formulation. A commercial graft copolymer composed of vinyl chloride, ethylene and vinylacetate was reacted with sodium N,N-diethyl-dithiocarbonate to introduce reactive dithiocarbamate groups. This dithiocarbamate derivatized hydrophobic copolymer was then copolymerized with methoxypoly-ethyleneglycol methacrylate, a hydrophilic monomer, and N,N-dimethylaminoethyl methacrylate, a monomer with a quaternizable amine, by UV irradiation. The N,N-dimethylaminoethyl groups were subsequently quaternized by reaction with ethyl bromide to provide a final polymer containing hydrophobic, hydrophilic and cationic portions. The relative abundance of either component could be varied by varying monomer feed compositions to provide a wide range of swelling characteristics. Catheters, polymer films, tubings, etc. could be coated with the above cationic polymer by solvent casting from a mixture of cyclohexanone and dimethyl-formamide containing 2-3% of the cationic polymer. After casting, the polymer coatings were dried at 70^{o}C for 5 hours and the remaining solvent was extracted in methanol, which was subsequently evaporated. Finally, these materials were soaked in a 2% sodium heparin solution for 1-3 days to ionically bind heparin to the quaternary ammonium groups. The relative abundance of hydrophobic

and hydrophilic groups, including both methoxypolyethylenegylcol methacrylate and the cationic methacrylate as hydrophilic groups, as determined by elemental analysis and by bromide titrations of ionic nitrogen atoms, ranged from 79%:21% (hydrophobic:hydrophilic) to 68%:32% with corresponding equilibrium hydrations of final heparinized polymers ranging from 12% to 44% water. These materials could bind as much as 19% by weight heparin, thus providing a large depot of heparin allowing high release rates over extended periods of time. Extensive *in vitro* and *in vivo* evaluations of these heparinized materials (64,65,66,67) have established a minimum heparin released rate of 4×10^{-2} $\mu g/cm^2/min$ as a critical parameter for nonthrombogenicity. This critical release rate could be provided for months *in vivo* by heparinized polymers with 15% by weight heparin and a hydrophobic:hydrophilic ratio providing 30% by weight equilibrium swelling in water.

Until the development of this heparin reservoir controlled release polymer, all other polymers previously sited provide heparin release only of surface bound heparin and the release kinetics were solely controlled by the ion exchange process. With the polymers developed by Tanzawa, et al. (64), heparin is first released from its cationic binding site by an ion exchange mechanism and then must diffuse through the polymer matrix to the surface where it is released into the blood or aqueous phase. With these polymers, the diffusion of heparin becomes the rate limiting step and therefore controls release kinetics. This rate limiting diffusion can then be controlled by the hydrophobic/hydrophilic ratio

of the polymer composition, providing an exquisite means for controlling heparin release.

Holland, et al. (68) have recently reported a convenient method for ionically binding heparin to hemodialysis membranes. By dissolving 5% diethylaminoethyl cellulose with 95% cellulose in appropriate solvents, cellulosic membranes are readily formed that contain cationic groups capable of ionically binding heparin. Heparin is released from these membranes and coagulation in contacting blood is controlled; however, platelets adversely interact with the membranes and platelet levels significantly decrease, compared to control cellulose membranes, with increased contact time.

A chronological summary of ionically bound, controlled release heparin polymers is presented in Table I.

1.4.2 Dispersed Heparin Polymers

A second means to provide heparinized polymers is to simply blend heparin throughout the polymer matrix, eliminating the need for cationic groups that may either leach off or remain as a permanent component of the polymer composition. By avoiding cationic groups, which can adversely affect platelets, improved *in vivo* blood compatibility can be expected.

Hufnagel, et al. (69) were first to incorporate heparin into either silicone rubber or a combination of silicone rubber plus colloidal graphite. Patches approximately 7 mm in diameter and 3 mm in thickness were fabricated with silicone rubber and silicone

Table I

Ionically Bound Heparin Material

Investigators	Material Treatment	Comments
Gott, et al.	Prior adsorption of a cationic surfactant onto graphite coated surfaces followed ionic heparin bonding.	Graphite surfaces are brittle and not applicable to most blood material applications. Heparin is released by an ion exchange mechanism.
Leininger, et al.	Formation of permanent quarternary amine groups in the polymer composition on the surfaces of various material followed by ionic heparin bonding.	Heparin is released by an ion exchange mechanism leading to eventual heparin depletion. Methods are applicable only to a limited number of materials.
Grode, et al.	Prior adsorbtion of TDMAC onto numberous preswollen biomedical materials followed by ionic heparin bonding.	Technique is relatively simple and applicable to numerous biomedical materials. Heparin is released by an ion exchange process leaving the TDMAC treated surface which adversely interacts with platelets.
Tanzawa, et al.	Formation of quarternary amine groups in the bulk copolymer preparation followed by ionic heparin bonding.	This heparinized copolymer provides a heparin reservoir that provides heparin release over longer periods of time. Heparin is initially released by an ion exchange process but heparin diffusion through the polymer is the rate limiting step. This copolymer can be grafted onto numerous biomedical materials.

rubber plus collodial graphite as control materials. Similar patches were prepared with heparin incorporated into silicone rubber and silicone rubber plus colloidal graphite ("Hepacone" and "Hepacone-G" respectively). When these materials were sutured onto the right atrial wall of mongrel dogs, both control materials were completely thrombosed within two hours while the Hepacone and Hepacone-G materials showed no thrombus formation even after 6 months.

Salyer and Weesner (70) blended heparin into eposy resins. Polymers containing 3 and 7 parts heparin per 100 parts of resin showed excellent nonthrombogenicity over a two week period of vascular implantation. These materials were shown to have greater initial plasma protein adsorption, when implanted in the venous system of dogs, than identical materials without heparin. However, after two weeks, the unheparinized materials were completely covered with thrombus while the heparin incorporated materials were clot free. These findings indicate that the incorporation of heparin into the polymeric materials influenced the adsorption of a benign protein layer; whereas without heparin, the material adsorbed proteins which presumably adversely interacted with platelets.

Although these authors initially thought the nonthrombogenicity of these materials was due to the slow leaching out of heparin into the blood phase, later studies by Salyer, et al. (71) showed that when heparin was combined with epoxy resin and urethane monomers, polymerization resulted in covalent incorporation

of heparin into the copolymer composition to form either poly-(heparin-ether) or poly(heparin-urethane) copolymers and heparin was not leaching out. In this study, the authors also investigated heparin ionically bound to permanent quaternary ammonium groups in the polymer composition. Epoxy and urethane polymers with chemically incorporated heparin demonstrated vastly increased whole blood clotting times relative to controlled polymers. Furthermore, these materials could be thoroughly washed and exposed to fresh blood for 30 minute periods, repeating this process for up to 26 times, and no clotting was observed. Implantation into the venous system of dogs showed no thrombus formation for up to 6 months.

A novel approach to the controlled release of heparin from polymer matrices was provided by Ebert, et al. (72). As previously stated, heparin can adversely interact with platelets, resulting in aggregation and potentiation of aggregation and release reactions caused by exogenous agents. Prostaglandins (PGE_1, PGI_2, PGD_2) are agents known to prevent platelet aggregation and degranulation by stimulating membrane bound adenyl cyclase, resulting in increase intracellular c-AMP levels (73,74,75). By combining both heparin and prostaglandin into controlled release polymer matrices, both intrinsic coagulation and adverse platelet interactions could be controlled. When heparin and PGE_1 were simultaneously combined with dissolved poly-hydroxyethyl methacrylate, polymers were formed where heparin and PGE_1 were released over approximately a 10 hour period. Bioassays of both heparin

and PGE_1 showed that neither agent had decreased biological activity resulting from polymer fabrication techniques.

These materials inhibited blood coagulation and platelet adhesion via separate biological mechanisms; however, due to the quick release rate, they would not be applicable to long term applications. A summary of physically dispersed, controlled release heparin polymers is presented in Table II.

1.5 Covalently Immobilized Heparin Materials

A disadvantage with the ionically bound and physically dispersed heparin/polymer systems is that heparin is continually depleted with time, thereby limiting the effective anticoagulant duration of such materials. Numerous investigators have covalently bound heparin to polymer surfaces to provide long term heparinized materials.

Grode, et al. (76) in addition to reporting progress on TDMAC ionically heparinized materials, reported the covalent coupling of heparin to APTES treated silicone rubber via a heparin/cyanuric chloride adduct. The authors also reported a procedure for radiation grafting polystyrene to various polymeric materials. The resultant polystyrene surfaces were then chloromethylated and subsequently treated with an ammonia/alcohol solution to form benzylamine groups. These polystyrene/benzylamine surfaces were then heparinized via the heparin/cyanuric chloride adduct. Heparin attachment was stable, even in 4N NaCl, and the authors report that both treatments resulted in materials that prolonged whole blood

Table II

Physically Dispersed Heparin Materials

Investigators	Material Treatment	Comments
Hufnagel, et al.	Heparin dispersed in silicone rubber and silicone rubber plus graphite.	Heparin released via diffusion from the polymer matrix provides thromboresistant materials, but affects of heparin dispersion on the mechanical properties of the polymers were not investigated.
Salyer and Weesner	Heparin dispersed in epoxy resins and urethane monomers prior to polymerization.	Although these investigators initially thought that heparin was leaching out, follow up studies showed that the heparin was covalently incorporated into the polymer composition.
Ebert, et al.	Heparin and prostaglandin physical dispersed into p-HEMA polymers.	Active heparin and antiplatelet prostaglandin are simultaneously released over an approximate 10 hour period. This material would only be applicable for acute applications.

clotting times. In vivo venous implantation tests (77) showed that cyanuric chloride/heparin adduct covalently coupled to APTES treated silicone rubber produced materials that prevented thrombus formation for up to 6 months.

Merrill, et al. (78) covalently coupled heparin to polyvinyl alcohol via glutaraldehyde crosslinking in the presence of an acid catalyst through hydroxyl groups on heparin and polyvinyl alcohol. Using ^{35}S-labeled heparin covalently coupled to polyvinyl alcohol, numerous clotting tests were conducted including thrombin time, partial thromboplastin time, activated partial thromboplastic time, prothrombin time and whole blood clotting time. In all cases, clotting times were increased following plasma or whole blood, depending upon the test, exposure with the covalently heparinized material. Sulfur-35 analyses of resultant plasmas showed no heparin to be present, eliminating the possibility that prolonged clotting times were due to heparin release. Pretreatment of the heparinized materials with albumin resulted in thrombin times paralleling control material. This lead the authors to the conclusion that specific coagulation factors were interacting with the immobilized heparin, resulting in coagulation factor deficient plasma, thereby producing prolonged clotting times. Pretreatment with albumin therefore covered heparin binding sites for coagulation factors. Coagulation factor assays on plasma exposed for 15 minutes to the heparinized material demonstrated up to a 59% decrease in factor XI, a 44% decrease in factor IX and a 29% decrease in factor V.

When platelet rich plasma was exposed to heparinized polyvinyl alcohol, for 20 minutes, platelet levels decreased 29.8%; however, since untreated polyvinyl alcohol and glutaraldehyde treated polyvinyl alcohol materials were not evaluated, conclusions regarding platelet interaction with the immobilized heparin cannot be made.

Lagergren and Eriksson (79) first reported the preparation of a crosslinked heparin monolayer surface. By first adsorbing a cationic surfactant, surfactants with 16-18 carbon atoms worked best, onto a polypropylene surface, cationic surface groups were introduced that could bind heparin as with the numerous ionically bound heparin polymers. The heparin molecules ionically bound to the cationic surface sites were then crosslinked with glutaraldehyde to provide a covalently stabilized heparin monolayer. When blood was exposed to such surfaces no clotting was observed even after 3 hours of contact; however, when the same blood was then subsequently placed in contact with glass, clotting was observed in 9 minutes.

Arteriovenous shunts were prepared with 3 mm ID polypropylene tubing with the crosslinked heparin monolayer, control shunts consisted of untreated polypropylene. When placed between the femoral artery and vein of mongrel dogs, the heparin monolayer shunts remained open for over 12 hours while the untreated polypropylene shunts completely thrombosed within 7-12 minutes.

This group has since published several papers on the preparation of heparin crosslinked monolayers with various substrate

materials and their evaluations. In general, the heparin monolayer is stabled, approximately 3% of the total affixed heparin is released over an initial 10 hour period and essentially no release occurs thereafter (80). Blood exposed to such surfaces demonstrates variable prolonged clotting times depending upon the degree of glutaraldehyde crosslinking. This has been described by Larsson, et al. (81) to be related to the ability of the affixed heparin to migrate from the surface and become incorporated into the surface adsorbed plasma proteins. By use of Auger spectroscopy, Eriksson, et al. (82) have shown sulfur (i.e., heparin) to be present on the surface of the adsorbed protein layer which they take as evidence for the above hypothesis. Finally, platelet compatibility is dramatically improved relative to untreated control materials (83).

Schmer (84) covalently coupled heparin to agarose by three methods: (1) direct coupling of heparin to cyanogen bromide activated agarose; (2) coupling of heparin to putrescine on putrescine derivatized agarose via thiophosgene and; (3) covalent coupling of heparin to amine groups on ε-amino caproyl of ε-amino caproyl derivatized agarose via a water soluble carbodiimide. The first method represents essentially direct coupling to the surface, the imidocarbonate group formed via the cyanogen bromide activated groups being rather small. The second and third methods interpose a spacer arm between the surface and the affixed heparin. Not only were greater quanities of heparin immobilized onto the spacer arm derivatized agaroses, but the ability of the immobilized hep-

arin to bind ATIII was also increased. No differences were distinguishable in both coupling yields and ATIII binding for heparin coupled via the thiophosgene reaction or the carbodiimide reaction. In both reactions, heparin was coupled via primary amine groups on heparin to the appropriate spacer arm.

Labarre, et al. (85) described the preparation of a heparin-methyl methacrylate copolymer. The reaction between hydroxyl groups on poly alcohols (i.e., heparin) with cerium (IV) salts in acidic aqueous solutions is known to produce free radicals capable of polymerizing vinyl monomers. By dissolving heparin in a 0.25 M nitric acid solution, to which an ammonium cerium (IV) nitrate and methyl methacrylic monomer are serially added, an insoluble heparin-methyl methacrylate copolymer is formed. Due to the numerous hydroxyl groups present on a heparin molecule, this copolymer is most likely a block copolymer consisting of a heparin core from which poly-methyl methacrylate chains extend. These heparin-MMA copolymers show a dose related anticoagulant activity verified by both plasma recalcification time and thrombin time assays. Later studies (86) showed these increased clotting times were due, in part, to noncovalently bonded heparin released from the insoluble heparin-MMA copolymer. Pretreatment of the copolymer with a sodium citrate solution removed that nonbound heparin, presumably by chelating cerium (III) ions formed during the copolymerization reaction, to which heparin was ionically binding. These washed copolymers also prolong thrombin times, both when evaluating plasma-copolymer suspensions and supernatants obtained

by centrifugation of plasma-copolymer suspensions, in a dose related manner.

Danishefsky and Tzeng (87) reported the preparation of a heparin-agarose polymer, where heparin was linked via an aminoethyl spacer arm. Aminoethyl agarose was mixed with a heparin solution, the pH was lowered to 4.75 and a water soluble carbodiimide was added to form an amide bond between heparin carboxylic groups and amine groups on the derivatized agarose. The reaction took place initially for 6 hours at room temperature, followed by approximately 18 hours at $4^{o}C$. The heparinized gels were washed with concentrated NaCl and stored several months. No leaching of heparin was observed during the storage. One ml columns were prepared with the heparinized gel and citrated plasma was eluted through each column, sequential fractions were collected. The first fractions did not clot upon recalcification for over one hour and activated partial thromboplastin times were in excess of 25 minutes. Subsequent fractions clotted upon recalcification within 15 minutes, while final fractions showed recalcification times equivalent to untreated plasma. When the columns were thoroughly washed with 4 M NaCl and reequilibrated with 0.9% NaCl, subsequent addition of plasma again produce plasma fractions with prolonged clotting times, indicating saturation of heparin binding sites for coagulation factors. Washing the plasma treated columns with 0.4 M and 1.2 M NaCl eluted fractions containing factors IX and XI respectively, as determined by the addition of 0.4 M NaCl and 1.2 M NaCl elutants to factor IX and factor XI deficient

plasmas. Additional assays for ATIII revealed that the immobilized heparin was also binding ATIII, only to a smaller extent than factors IX and XI.

Goosen and Sefton (88) reported the synthesis of a heparinized styrene-butadiene-styrene (SBS) elastomer. The double bonds of the butadiene components of the SBS rubber were first hydroxylated by reaction with peracetic acid. By controlling reacting conditions the process could be limited to the SBS polymer surface, thereby preserving the elastomeric bulk properties of the material. After cleaning the surfaces by either glow discharging or ultrasonic cleaning, the hydroxylated SBS films were dipped in a solution containing polyvinyl alcohol, formaldehyde, glutaraldehyde, glycerol, magnesium chloride, sodium heparin and water. A resultant lamination occurs where the hydroxyl groups on the SBS surface bond via acetal bridges to the hydroxyl groups of heparin. This procedure results in a tightly adhering PVA laminated SBS rubber with heparin covalently immobilized to the PVA surface. When plasma was exposed to these surfaces, partial thromboplastin times were greatly elevated, eluted ^{35}S-heparin was not detectable in the plasma. Similarily, A-V silastic shunts coated with the heparinized SBS material remained nonthrombosed for extended durations relative to nonheparinized SBS coated shunts. When whole blood was incubated inside heparinized shunts, no clots were observed after 45 minutes. Placing that same blood in contact with glass resulted in subsequent clotting within 5 minutes. Platelet adhesion testing, however, revealed significantly increased adhe-

sion for heparinized materials relative to unheparinized controls.

Muria, et al. (89) have immobilized heparin to a variety of cyanogen bromide activated surfaces including sepharose, polyhydroxyethyl methacrylate (P-HEMA) and polyvinyl alcohol. Heparin immobilized on sepharose or P-HEMA greatly prolonged plasma recalcification times while heparin immobilized on PVA did not greatly effect plasma recalcification times. Thrombin is instantaneously neutralized in the presence of soluble heparin and ATIII; however, thrombin neutralization by immobilized heparin was indistinguishable in the presence or absence of ATIII. This finding would indicate that under these immobilization conditions, utilizing amine groups on heparin, immobilized heparin was not able to interact with ATIII in the same manner as it does with soluble heparin.

This could also be interpretated as evidence that heparin acts by first binding to thrombin, potentiating ATIII binding to a heparin/thrombin complex. Recent studies by Goosen and Sefton (90) using polyvinyl alcohol immobilized heparin, prepared according to the methods of Merrill, et al. (78), demonstrated that immobilized heparin neutralized thrombin by first binding thrombin to form a heparin/thrombin complex that further binds ATIII.

With both of these systems, heparin is immobilized in close proximity to the polymer surface. Schmer (84) demonstrated that heparin immobilized by a cyanogen bromide reaction, identical to the methods of Muira (89), did not bind ATIII as effectively as heparin immobilized via a spacer arm separating the affixed heparin from the surface. A possibility that neither Muira or Goosen

have addressed, is that their immobilized heparin systems do not allow interactions between immobilized heparin and ATIII similar to interaction between soluble heparin and ATIII. The ATIII binding sites on the immobilized heparin may not have been accessible to ATIII.

A summary of immobilized heparin polymers is presented in Table III.

1.6 Hypothesis for Immobilized Heparin Activity

When blood contacts an immobilized heparin surface proteins are adsorbed. If those adsorbed proteins included coagulation factors, *in vitro* clotting times may be increased. In any event, the nature of those adsorbed proteins can influence platelet interactions with the surface during *in vivo* usage and the presence of the highly anionic heparin would be expected to possibly alter the nature of those adsorbed proteins relative to the initial control surface.

Previous work with immobilized heparin surfaces has shown that these materials adsorb coagulation factors and thereby prolong *in vitro* clotting times; however, does the heparin immobilized onto these material interact similar to soluble heparin or are the prolonged *in vitro* clotting times simply the manifestation of plasma protein interactions with a negatively charged surface?

Past investigators have immobilized heparin via several functional groups on the heparin molecule (amine, hydroxyl and carboxylic functional groups) yet none of those studies have estab-

Table III

Covalently Immobilized Heparin Materials

Investigators	Material Treatment	Comments
Grode, et al.	Covalent coupling of a heparin/cyanuric chloride adduct to APTES treated silicone rubber.	Produces a permanent heparinized surface; however, the effects of forming the heparin/cyanuric complex on the anticoagulant activity were not investigated.
Merrill, et al.	Glutaraldehyde crosslinking of heparin to polyvinyl alcohol.	Produces a permanent crosslinked heparin surface that adsorbs coagulation factors from plasma. Platelet function is worse for the heparinized materials than for the initial polyvinyl alcohol. The effects of heparin crosslinking on the anticoagulant activity were not investigated.
Lagergren, et al.	Prior adsorption of a cationic surfactant followed by ionic heparin binding and subsequent heparin crosslinking.	Effects of heparin crosslinking on the anticoagulant activity were not evaluated. Platelet interactions were improved for heparinized materials relative to starting materials.
Schmer	Heparin coupled to surfaces via spacer arms.	Heparin coupled via spacer arms was shown to interact with ATIII better than heparin directly coupled to surfaces.
Muira	Heparin directly coupled to cyanogen bromide activated surfaces.	Immobilized heparin does not follow soluble heparin behavior. Thrombin neutralization was indistinguishable in the presence or in the absence of ATIII.

lished the effects of derivatizing those groups on the heparin anticoagulant activity. It is entirely possible that blocking those groups in reactions similar to the immobilization reactions could result in the loss of anticoagulant activity and one would then conclude that heparin immobilized via those groups would also not be active.

Another unanswered question lies in the protein adsorption/ platelet interaction phenomena. Do platelets actually contact immobilized heparin or do the adsorbed plasma proteins mask the heparinized surface? In the first case, immobilized heparin could adversely affect platelets similar to soluble heparin interactions with platelets, while in the latter case, the nature of the adsorbed plasma proteins would dictate platelet interactions and immobilized heparin would be indirectly affecting platelets by influencing the nature of the adsorbed plasma proteins.

As previously stated, soluble heparin inhibits thrombin activity and the activity of other coagulations factors presumably via antithrombin III. The majority of past work would indicate that immobilized heparin does not interact with ATIII as does soluble heparin, thrombin neutralization by immobilized heparin being indistinguishable in the presence or absence of ATIII being one example (89). However, Schmer, et al. (84) have shown that immobilized heparin can better interact with ATIII if the heparin was immobilized via a spacer arm.

Work in this dissertation is divided into four major categories:

(1) Heparin derivatization to determine changes in anticoagulant potency associated with the covalent modification of specific functional groups, thereby determining which functional groups should participate in immobilization reactions.

(2) The effects of spacer arm length on immobilized heparin anticoagulant activity.

(3) Platelet interactions with immobilized heparin.

(4) Evaluation of adsorbed protein surface interactions with heparin immobilized surfaces.

CHAPTER 2

METHODS

2.1 Functional Group Derivatizations

2.1.1 Amine Group Derivatization

Free amine groups on porcine intestinal sodium heparin (Sigma Chemical Company) were blocked with acetic anhydride (91) by the following procedures. Three and one quarter grams of sodium heparin, activity - 153 USP units/mg, were dissolved in 300 ml of distilled water (pH - 6.29) to which 1.63 grams of $NaHCO_3$ were added (pH - 8.40). A USP unit of activity is defined as a plasma heparin activity producing 50% clotting in citrated sheep plasma one hour after the addition of excess calcium chloride. This solution was cooled to 0^oC in an ice bath. Reagent grade acetic anhydride (American Scientific and Chemical Company) was added to acetone, previously distilled and collected over 3A^o molecular seives (MCB Manufacturing Chemists, Inc.) to remove water; a total of 6.54 ml acetic anhydride was added to 32.7 ml acetone. This acetic anhydride solution was added drop wise over 40 minutes to the 0^oC heparin solution while constantly stirring and maintaining the pH between 8.0 and 8.4 by the addition of 1 M Na_2CO_3. After the addition of the acetic anhydride, the reaction was brought to room temperature for 2.5 hours while maintaining the pH between 8.0 and

8.4 with the addition of 1 M Na_2CO_3. The reaction contents were then dialyzed at 2°C in 2000 MW cut off dialysis tubes (Spectrum Chemical Company) with distilled water for 3 days; changing the dialysate, total volume 5 liters, twice a day. After the final dialysis, the heparin solution was first concentrated by roto-evaporization at 35-40°C and freeze dried.

2.1.2 Carboxylic Group Derivatization

Since crosslinking between free amine groups on heparin with activated carboxylic groups on heparin formed during the carboxylic group derivatization reactions was a concern, N-acetylated heparin, which was prepared as described in the previous section, was used in all carboxylic group derivatization studies. Two grams of N-acetylated heparin and 2.0 ml of n-butylamine were dissolved in 40 ml of distilled water and the reaction vessel was placed in a cold room at approximately 2-4°C. The pH was adjusted to 4.75 with 1 M HCl and a total of 0.8 grams of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (Sigma Chemical Company) was added to the heparin/n-butylamine solution in approximately 10 to 20 mg portions over a 6 hour period while constantly stirring.

Periodic samples were withdrawn from the reaction vessel during the 6 hour period and dialyzed at 2°C with 2000 MW cut off dialysis tubes for 2 days in 1 M NaCl, changing the 5 liter dialysate volume twice each day. Finally, the samples were dialyzed with distilled water for 1 day, changing the dialysate twice. The

samples were concentrated by roto-evaporization at 35-40^{o}C and freeze dried.

In a separate reaction, 0.5 grams of N-acetylated heparin and 1 gram 2-aminoethyl hydrogen sulfate (Aldrich Chemical Company) were dissolved in 10 ml of distilled water and the reaction was placed in the cold room at approximately 2-4^{o}C. The pH was adjusted to 4.75 with 1 M HCl and a total of 0.2 grams of EDC were added in approximately 10 to 20 mg portions over a 4 hour period while constantly stirring. The reaction contents were then dialyzed in 2000 MW cut off dialysis tubes with 1 M NaCl for 2 days, changing the 5 liter dialysate volume twice a day, followed by 1 day of dialyses with distilled water, changing the dialysate twice. The sample was then concentrated by roto-evaporatization at 35-40^{o}C and freeze dried. The carbodiimide reaction scheme (92) is presented in Figure 4.

2.1.3 Hydroxyl Group Derivatization Epichlorohydrin Reactions

As with the carboxylic group derivatization reactions, all hydroxyl group derivatization reactions were conducted with N-acetylated heparin to prevent intra- and intermolecular cross-linking. One half gram of N-acetylated heparin was first dissolved in 1.0 M Na_2CO_3 and then placed in a 40^{o}C water bath. One milliliter of epichlorohydrin (Aldrich Chemical Company) and either 1 ml of n-butylamine (Aldrich Chemical Company) or 1 gram of glycine (Aldrich Chemical Company) or 2-aminoethyl hydrogen sulfate were then added to the reaction vessel, the resultant

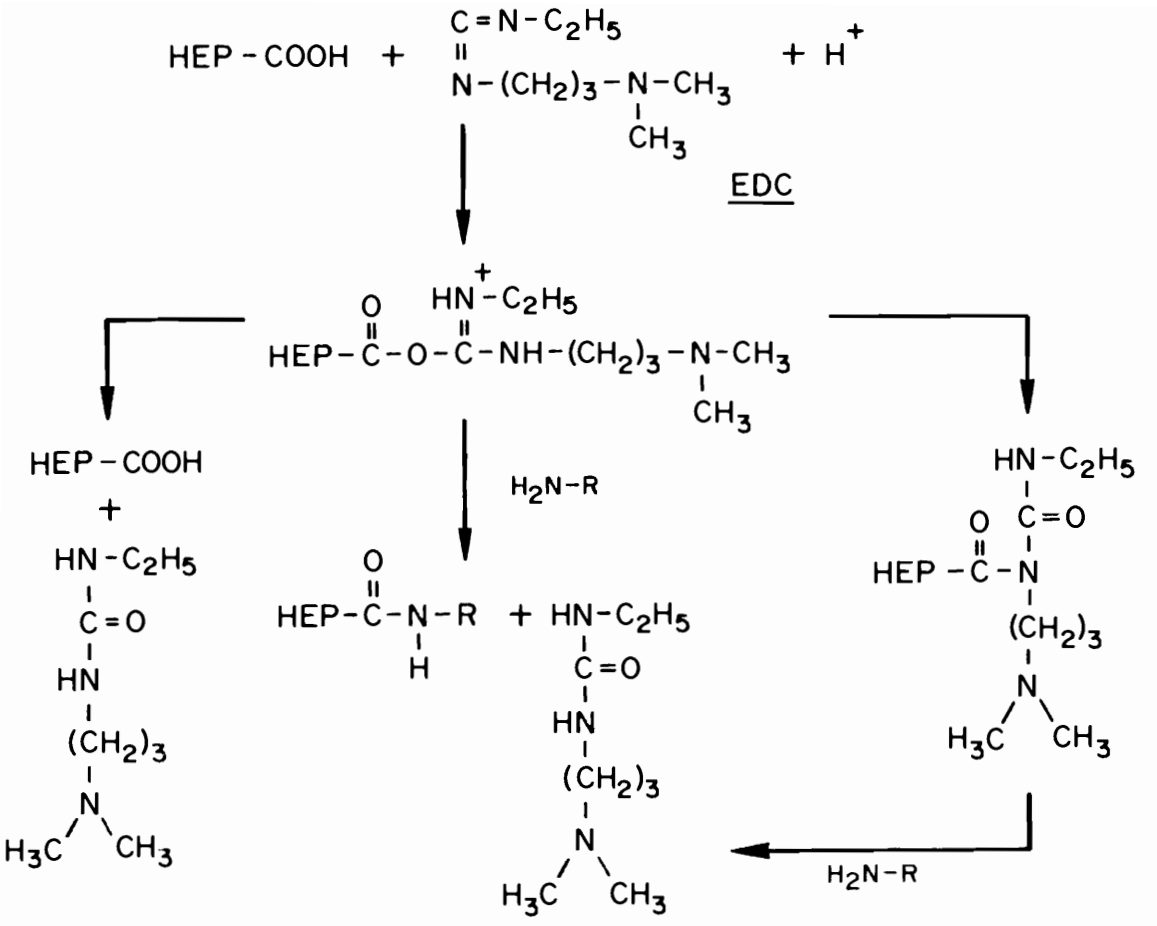


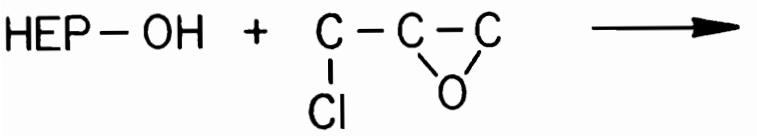
Figure 4. Carbodiimide Reaction Scheme

emulsion being constantly stirred. After 5 hours of reaction time at 40°C, the reaction samples were transferred to dialysis tubing and dialyzed against 1 M NaCl for 2 days, changing the 5 liter dialysate twice a day, followed by 1 day dialysis against distilled water, which was also changed twice. The hydroxyl group derivatized heparin samples were concentrated by roto-evaporization and finally freeze dried. The epichlorohydrin reaction scheme is shown in Figure 5.

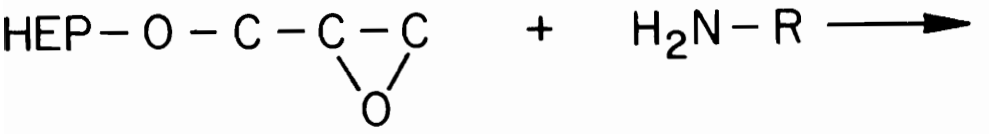
2.1.4 Hydroxyl Group Derivatization Divinylsulfone Reactions

One half gram of N-acetylated heparin was dissolved in 10 ml of 1.0 M Na_2CO_3 solution. One milliliter of divinylsulfone (Aldrich Chemical Company) and either 1 ml of n-butylamine or 1 gram of either glycine or 2-aminoethyl hydrogen sulfate were added to the heparin solution to form an emulsion which was constantly stirred at room temperature for 3 hours. The reaction contents were then dialyzed in 2000 MW cut of dialysis tubes for 2 days in 1 M NaCl followed by 1 day dialysis in distilled water; the 5 liter dialysate volume, either 1 M NaCl or distilled water, was changed twice each day. After dialysis, the samples were concentrated by roto-evaporization and freeze dried. The divinylsulfone reaction scheme is presented in Figure 6.

HYDROXYL DERIVATIZATION
(EPICHLOROHYDRIN)



EPICHLOROHYDRIN



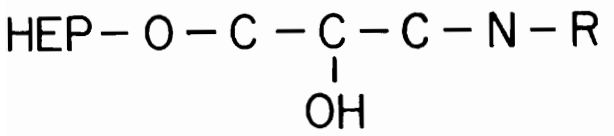


Figure 5. Epichlorohydrin Reaction Scheme

HYDROXYL DERIVATIZATION
(DIVINYLSULFONE)

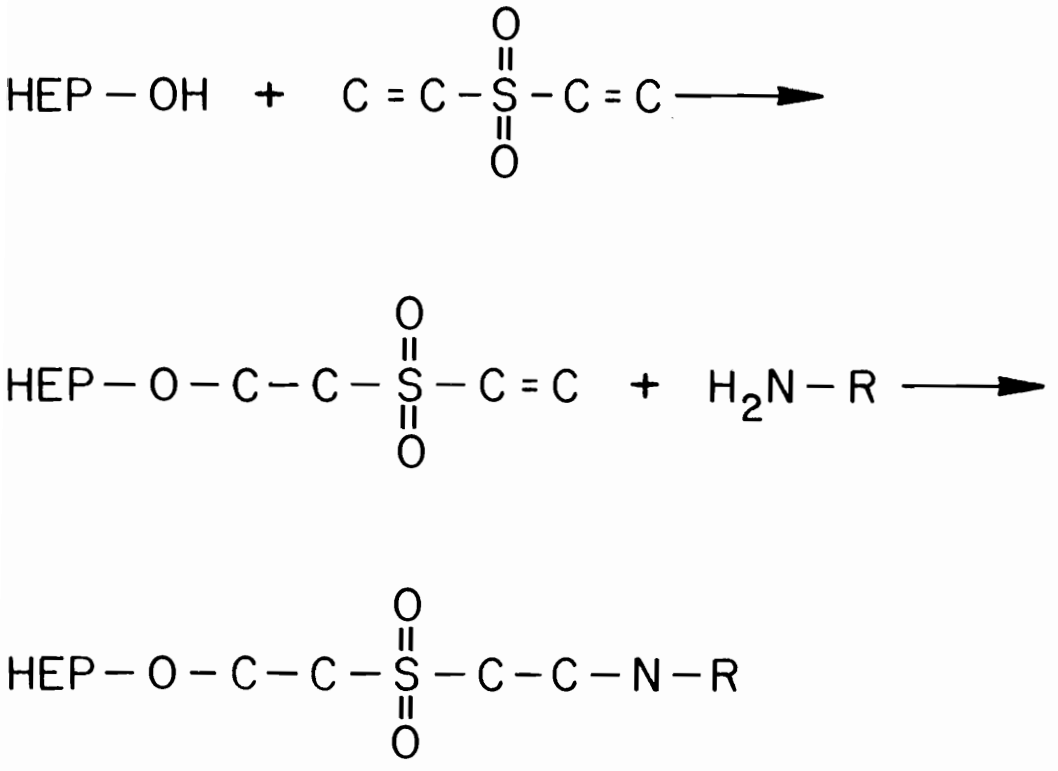


Figure 6. Divinylsulfone Reaction Scheme

2.2 Polymer Substrate Preparation for Heparin Immobilization

2.2.1 Diaminoalkane-Agaroses

Diaminoalkane-agaroses (Sigma Chemical Company) were washed with pH 7.4 phosphate buffered saline (PBS) and used without further treatment. These materials were used in *in vitro* coagulation studies due to the high surface areas obtained for that bead diameter (∿ 100 μm). The diaminoalkane derivatized agaroses were as follows: 1,2 diaminoethane-agarose, 1,4 diaminobutane-agarose, 1,8 diaminooctane-agarose, 1,10 diaminodecane-agarose and 1,12 diaminododecane-agarose.

2.2.2 Diaminoalkane Coupling to Sepharose 6-MB Beads

All platelet interaction studies were conducted on Sepharose 6-MB (Pharmacia Fine Chemicals, Inc.) derivatized beads. These beads provide greater surface areas for platelet interaction studies compared to flat surfaces; however, these beads are substantially larger (mean diameter ∿ 400 μm) than the agarose beads used in *in vitro* coagulation testing (∿ 100 μm mean diameter). The Sepharose 6-MB, because of their larger size, are more suited to column type platelet interaction studies than small beads which provide interstitial gaps too small for blood cells to pass through. Diaminoalkane derivatized Sepharose 6-MB beads were prepared with CNBr activated Sepharose 6-MB (purchased as CNBr activated beads). These beads were obtained in a dehydrated state containing agents to preserve the pore structure and bead shaped

following dehydration. Ten grams of the dehydrated Sepharose 6-MB beads were first hydrated in 2 liters of 0.02 M HCl for 30 minutes to hydrate the beads and extract the above additives as per the manufacture's recommendation. If this first step is not followed, subsequent coupling reactions are greatly diminished. The 0.02 M HCl hydrated beads were then suction dried on a sintered glass funnel and rehydrated in 0.5 M Na_2CO_3 for 2 hours, the solution to be used in subsequent coupling reactions. The beads were then suction dried again and divided into two portions which were added to either 3.0 grams of diaminobutane dissolved in 100 ml of 0.5 M Na_2CO_3 or 3.0 grams of diaminododecane dispersed in 100 ml of 0.5 M Na_2CO_3, due to the low aqueous solubility of diaminododecano, in closed vessels. The vessels were rotated at approximately 1 revolution per second for 4 hours at room temperature (stirring will destroy the bead structure), and then stored at 2^oC for 24 hours. The diaminoalkane derivatized beads were then repeatedly washed with pH 7.4 phosphate buffered isotonic saline and suctioned dried with a sintered glass funnel. The beads, still hydrated, were then placed in 100 ml of 2 M ethanolamine in 0.5 M Na_2CO_3 solution for 24 hours at 2^oC to block any possible remaining CNBr activated sites. The beads were then again repeatedly washed with pH 7.4 phosphate buffered isotonic saline and stored at 2^oC for 1 month in the buffered saline solution.

2.2.3 Diaminoalkane Coupling to Cellulose Sheets

Cuprophan$^{(R)}$ a commercially available regenerated cellulose,

was obtained from a Gambro lundia major(R) hemodialyzer (Gambro Inc.) and was the polymer substrate used for all "flat sheet" immobilized heparin evaluations. To remove glycerol, Cuprophan was first washed by soxhlet extraction with double distilled water for 24 hours. The Cuprophan was then cyanogen bromide activated by reacting a total of 250 cm^2, divided into $\sim$ 1 X 2 cm^2 pieces, with 10 gms of CNBr (Aldrich Chemical Company) dissolved in 400 ml of 0.5 M Na_2CO_3 at a pH of 11.3 for 10 minutes. The CNBr activated cellulose sheets were then thoroughly washed with distilled water and divided into portions which were then reacted with either 3.0 grams of diaminobutane (Aldrich Chemical Company) in 100 ml of 0.5 M Na_2CO_3 or with 3.0 grams of diaminododecane (Aldrich Chemical Company) dispersed in 100 ml of 0.5 M Na_2CO_3, due to the low aqueous solubility of the diaminododecane, for 4 hours at 22°C followed by 24 hours at 2°C.

The above diaminobutane and diaminododecane derivatized cellulose sheets were extensively washed with pH 7.4 phosphate buffer isotonic saline and possible remaining CNBr activated sites were blocked by placing the diaminoalkane derivatized sheets in 100 ml of 2 M ethanolamine in 0.5 M Na_2CO_3 solution at 2°C for 24 hours. The diaminobutane and diaminododecane derivatized cellulose sheets were then extensively washed again in pH 7.4 phosphate buffered isotonic saline and stored 1 month in pH 7.4 PBS at 2°C. The CNBr activation and diaminoalkane coupling reaction schemes are presented in Figure 7.

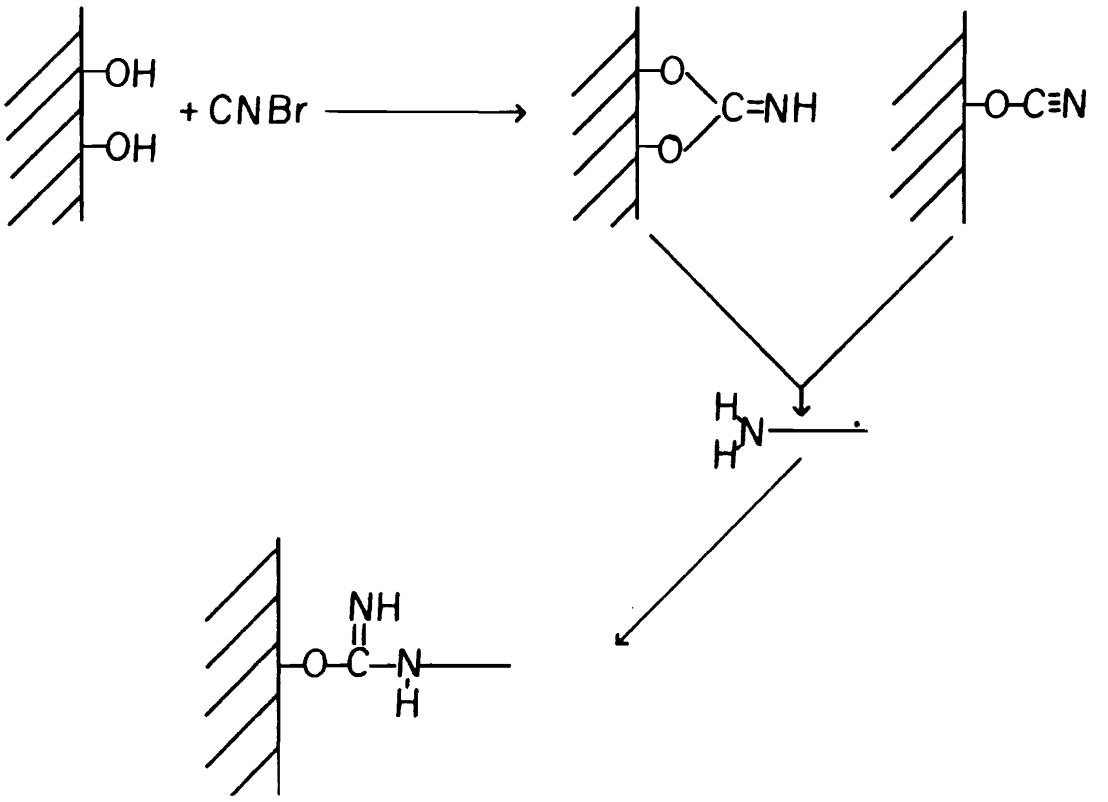


Figure 7. Cyanogen bromide activation and diaminoalkane coupling scheme.

2.3 Quantitation of Degrees of Diaminoalkane Coupling to Polymer Substrates

2.3.1 Preparation of ^{14}C-Sodium Acetate

Carbon-14 labeled sodium acetate (New England Nuclear) was mixed with a solution of cold anhydrous sodium acetate (Aldrich Chemical Company) in doubled distilled water and freeze dried. Weighed samples of the resultant diluted ^{14}C-sodium acetate were dissolved in water, portions of which were then dissolved in liquid scintillation fluid and counted for carbon-14. The above ^{14}C-sodium acetate had a specific activity of 202,191 $\pm$ 1,236 dpm/mg. Carbon-14 levels in blank scintillation fluid ranged from 10-20 cpm, 15 cpm was therefore subtracted from all C-14 samples and respective dpms were recalculated.

2.3.2 Determination of Diaminoalkane Coupling to Agarose Beads

The degrees of diaminoalkane coupling to agarose gels was conducted by the manufacturer and was supplied with each gel.

2.3.3 Determination of Diaminoalkane Coupling to Sepharose 6-MB - Sodium Acetate Coupling

The methods for coupling ^{14}C acetate to the diaminoalkane derivatized Sepharose 6-MB were analogous to the methods in Section 2.1.2 above. Known packed gel volumes of hydrated diaminobutane and diaminododecane derivatized Sepharose 6-MB ($\sim$ 2 cc), after

one month storage and subsequent washing, were added to a 20 ml solution containing 190 mg ^{14}C-sodium acetate plus 822 mg of EDC, approximate molar ratio 1:2. The pH was adjusted to 4.75 and the reaction proceeded at 2-4^{o}C for 24 hours. The gels were then repeatedly washed with pH 7.4 buffered saline until C-14 was not detected above background in the wash solutions. The ^{14}C-acetate coupled beads were then vacuum dried.

Three milliliters of concentrated sulfuric acid was then added to hydrolytically cleave the glycosidic bonds and 3.0 ml of ethanol was then added to each solution. Two hundred and fifty microliters of each solution was then counted for carbon-14. Each acid dissolved polymer was counted in triplicate. For determining the specific activity of the immobilized ^{14}C-acetate the specific activity of the sodium acetate had to be adjusted to include only the acetate portion. The specific activity of the acetic acid coupled to the diaminoalkane derivatized Sepharose 6-MB was then 271,798 $\pm$ 1,662 dpm/mg.

2.3.4 Coupling of ^{14}C-Sodium Acetate to Diaminoalkane Cellulose Sheets

The degree of diaminobutane and diaminododecane coupling to the CNBr activated cellulose sheets was determined by coupling ^{14}C-sodium acetate to the terminal primary amines of the derivatized cellulose with EDC as described above. Known surfaces areas ($\sim$ 30 cm^2) of hydrated diaminobutane and diaminododecane derivatized cellulose sheets, after one month storage, were added

to a 20 ml solution containing 190 mg ^{14}C-sodium acetate plus 822 mg of EDC in distilled water. The pH was lowered to 4.75 and the reaction was conducted for 24 hours at 2-4^{o}C. The reaction scheme is analogous to that depicted in Figure 4. After 24 hours reaction time, the ^{14}C-acetate coupled diaminoalkane derivatized cellulose sheets were repeatedly washed with pH 7.4 phosphate buffered saline until no carbon-14 was detected above background in the wash solutions. The labeled cellulose sheets were then vacuum dried.

Three milliliters of concentrated sulfuric acid was then added to each labeled cellulose sheet to hydrolytically cleave the glycosidic bonds, 3.0 ml of ethanol was then added to each resultant solution. Two hundred and fifty microliters of each solution was then counted for carbon-14. Each acid dissolved cellulose was counted in triplicate.

2.4 Heparin Immobilization to Polymer Substrates

2.4.1 Heparin Immobilization to Diaminoalkane Derivatized Agarose Gels

In vitro coagulation tests were conducted with the heparinized agarose gels. Since any prolongation of clotting times could be the result of free heparin released from the heparinized gels, it was important that heparin levels in the test plasmas could be accurately determined. Therefore, all heparin immobilization to diaminoalkane derivatized agaroses were performed with tritium

labeled heparin.

Furthermore, carbodiimide reactions must be conducted under acidic conditions, typically near a pH of 4.75. At this pH, sulfate and sulfamate groups can hydrolyze over a 24 hour period, resulting in desulfunation with the concommitant loss of anticoagulant potency (93). Heparin was therefore immobilized to diaminoalkane derivatized surfaces with N-ethyl-5-phenylisoxazolium-3-sulfonate, Woodward's Reagent K (94). This reaction involves the ionized form of the carboxylic group and can be conducted over a wide range of pH's.

Tritium labeled sodium heparin (New England Nuclear) was added to a solution containing cold sodium heparin and was N-acetylated according to the procedures in Section 2.1.1. This ^{3}H-N-acetylated heparin had a specific activity of 43,114 $\pm$ 1,048 dpm/mg.

Sixty-eight milligrams of Woodward's Reagent K was added to a 100 ml solution of pH 7.4 phosphate buffered saline containing 8.0 mg/ml tritium labeled N-acetylated heparin and the carboxylic groups on the heparin were activated for 8 hours at 2-4^{o}C. This coupling step, similar to the carbodiimide reactions, entails the coupling of the Woodward's Reagent K to heparin carboxylic groups to form reactive intermediate compounds. Based on conductimetric titrations of heparin, to be discussed in detail in subsequent sections, heparin contains 1.68 X 10^{-6} moles of carboxylic functional groups per mg of heparin. The amount of Woodward's Reagent K added in all heparin immobilization reactions is a sufficient

quantity to activate a maximum of 20% of the total carboxylic groups. This activation of a maximum of 20% of the total carboxylic groups is of vital importance in retaining heparin activity and is discussed further in subsequent sections.

Ten milliliters of the above carboxylic activated, tritium labeled N-acetylated heparin solution was added to 4 ml of each diaminoalkane derivatized agarose. The mixtures were gently stirred for 24 hours at 4^{o}C. The Woodward's Reagent K reaction scheme for coupling heparin to primary amines on the diaminoalkane derivatized agarose is shown in Figure 8. The gels were then repeatedly washed with pH 7.4 phosphate buffered saline in 50 ml increments in a sintered glass funnel, collecting each washing for heparin quantitation by liquid scintillation counting of tritium levels in resultant wash solutions, a tritium background of 24.5 cpm was subtracted from all tritium samples. A total of twenty, 50 ml washings were used for each gel, typically heparin was not observed in wash solutions after the tenth washing.

The heparinized gels were then suction dried, still hydrated however, and 10 ml of 2 M ethanolamine was added to each heparinized gel to react any possible remaining Woodward's Reagent K activated carboxylic groups on the immobilized heparin. The gels were stored 24 hours at 2-4^{o}C in the ethanolamine solutions after which they were again repeatedly washed in pH 7.4 buffered saline, no heparin was detected in these wash solutions. The gels were stored in pH 7.4 buffered saline for further evaluations.

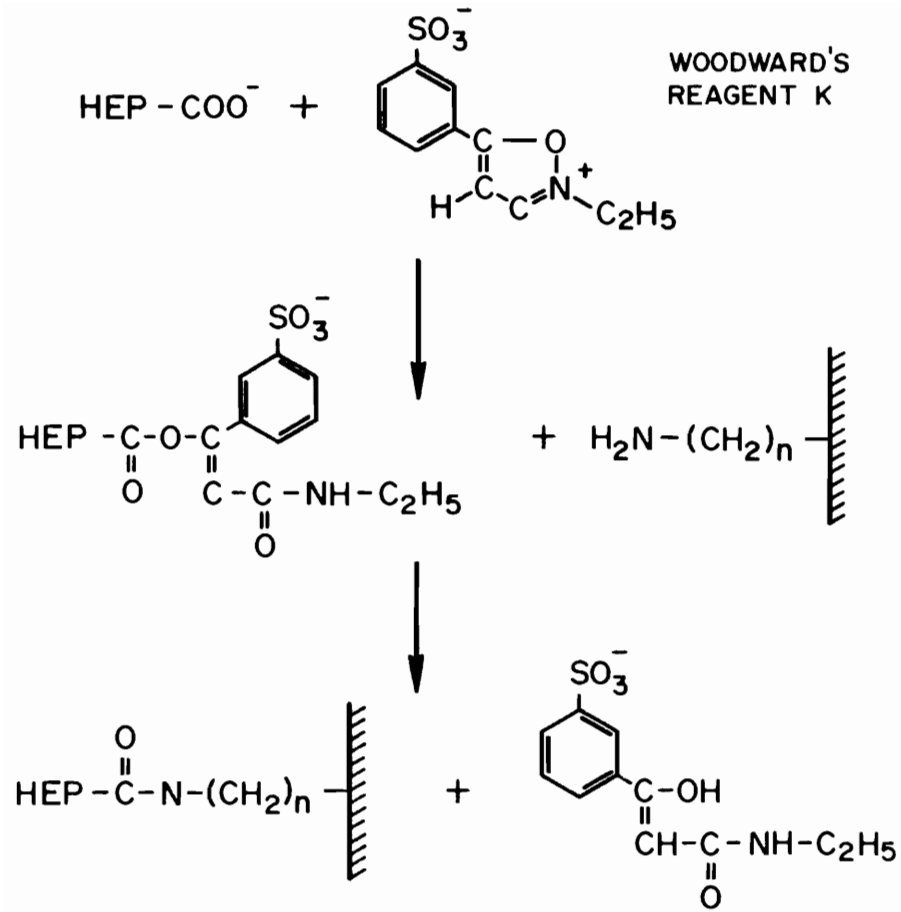


Figure 8. Heparin coupling to diaminoalkane derivatized substrates via Woodward's Reagent K.

2.4.2 Heparin Coupling to Diaminoalkane Derivatized Sepharose 6-MB

N-acetylated heparin was coupled to diaminobutane and diaminododecane derivatized Sepharose 6-MB in an analogous fashion to that described in Section 2.4.1. After one month storage and subsequent washing, N-acetylated heparin was coupled to the diaminoalkane derivatized Sepharose 6-MB beads via amide linkages using the condensing agent, Woodward's Reagent K. Sixty-eight milligrams of Woodward's Reagent K was added to a 100 ml pH 7.4 phosphate buffered saline solution containing 8.0 mg of non-labeled N-acetylated heparin per ml of solution. This is a sufficient quantity of Woodward's Reagent K to activate a maximum of 20% of the total carboxylic groups present in the 800 mg of N-acetylated heparin. The carboxylic groups were first activated for 8 hours at 2-4^{o}C. The activated heparin solution was then added to 3.0 grams of either suction dried diaminobutane or diaminododecane derivatized Sepharose 6-MB beads, which were then rotated in a closed vessel at approximately one revolution per second for 4 hours at 22^{o}C followed by 24 hours at 2-4^{o}C. The heparinized Sepharose 6-MB beads were then repeatedly washed in pH 7.4 phosphate buffered saline and added to 100 ml of 2 M ethanolamine in pH 7.4 phosphate buffered saline. The gels were then stored 24 hours at 2-4^{o}C in the ethanolamine solution to react any possible Woodward's Reagent K activated carboxylic groups on the immobilized heparin. The gels were then again repeatedly washed with pH 7.4 phosphate buffered saline and stored

stored at 2-4°C in buffered saline until further evaluation. Heparinized gels were not stored longer than 2 weeks under any circumstances. The immobilization of N-acetylated heparin to Sepharose 6-MB by Woodward's Reagent K is exactly analogous in that previously depicted in Figure 8.

2.4.3 Heparin Coupling to Diaminoalkane Derivatized Cellulose Sheets

Heparin was coupled to diaminobutane and diaminododecane derivatized cellulose sheets, after one month storage and subsequent washing, via an amide linkage, involving heparin carboxylic groups and the terminal primary amines on the derivatized cellulose using Woodward's Reagent K.

A 100 ml solution of pH 7.4 phosphate buffered saline, containing 8.0 mg/ml of nonlabeled N-acetylated heparin was first activated by adding 68.0 mg of Woodward's Reagent K. After adding the Woodward's Reagent K to the heparin solution, the carboxylic groups are first activated for 8 hours at 2-4°C.

The above solution was then added to approximately 25 cm^2 of either diaminobutane or diaminododecane derivatized cellulose sheets and allowed to react for 4 hours at 22°C followed by 24 hours at 2-4°C. The complete reaction scheme for the Woodward's Reagent K coupling of heparin to the primary amines of the derivatized cellulose is shown in Figure 8.

The immobilized heparin cellulose sheets were then washed with pH 7.4 phosphate buffered saline and 100 ml of 2 M ethanol-

amine was added to react any possible remaining Woodward's Reagent K activated carboxylic groups on the immobilized heparin. The heparinized cellulose sheets were stored at 2-4^{o}C in the ethanolamine solution for 24 hours, after which they were repeatedly washed with pH 7.4 phosphate buffered saline. The diaminobutane and diaminododecane mediated, immobilized heparin cellulose sheets were then vacuum dried and stored under vacuum dessication until needed for additional testing.

2.4.4 Heparin Immobilization to Sepharose 4-B Beads Via Divinylsulfone

Tritium labeled N-acetylated heparin was coupled to Sepharose 4-B beads via a divinylsulfone intermediate. Ten grams of suction dried Sepharose 4-B (Pharmacia Fine Chemicals, Inc.) was added to 10 ml of 0.5 M Na_2CO_3 with 1.2 ml of divinylsulfone and stirred for 2 hours at 22^{o}C. The above divinylsulfone activated Sepharose 4-B beads were then washed with phosphate buffered saline and suction dried with a sintered glass funnel. Four grams of the divinylsulfone activated Sepharose 4-B beads were added to 10 ml of 0.5 M Na_2CO_3 containing 100 mg of tritium labeled N-acetylated heparin, specific activity 43,114 dpm/mg, and the reaction contents were rotated at approximately one revolution per second for 24 hours. The gels were then repeatedly washed with pH 7.4 phosphate buffered saline in 50 ml increments in a sintered glass funnel, collecting each washing for heparin quantitation via liquid scintillation counting. A total of twenty, 50 ml washing

were used; no heparin was detected after the sixth washing. The divinylsulfone coupled heparinized Sepharose 4-B gels were stored in the pH 7.4 buffered saline until further evaluations. The divinylsulfone heparin immobilization reaction is analogous to Figure 6.

2.5 Quantification of Immobilized Heparin

2.5.1 Heparin Immobilized to Diaminoalkane Derivatized Agaroses

The quantity of heparin immobilized to the diaminoalkane agaroses were determined by the difference between the initial amount of tritium labeled N-acetylated heparin, specific activity 43,114 dpm/mg, added to each gel and the summation of the total amount of heparin present in the sequential 50 ml rinse solutions, see Section 2.4.1.

2.5.2 Heparin Immobilized to Diaminoalkane Derivatized Sepharose 6-MB

Tritium labeled sodium heparin was added to a solution of cold sodium heparin, which was then acetylated according to the procedures in Section 2.1.1. This N-acetylated heparin had a specific activity of 205,163 $\pm$ 807 dpm/mg. This tritium labeled N-acetylated heparin was then coupled to the diaminobutane and diaminododecane derivatized Sepharose 6-MB beads using the exact procedures described in Section 2.4.2. These tritium heparin coupling reactions were conducted in parallel with the cold heparin

coupling reactions using the same batches of diaminoalkane derivatized Sepharose 6-MB beads. After repeated washings, following the procedures described in Section 2.4.1, known volumes of the tritium labeled immobilized heparin gels (∿ 2.0 ml) were first dissolved with concentrated sulfuric acid to which ethanol was added, analogous to the procedures for determined degrees of diaminoalkane coupling to Sepharose 6-MB beads and cellulose sheets in Sections 2.3.2 and 2.3.3 respectively. Two hundred and fifty microliters of the above solutions were then added to 15 ml of scintillation fluid and tritium levels were counted. Each acid dissolved tritium labeled heparinized gel was counted in triplicate. A tritium background level of 24.5 cpm was subtracted from all tritium samples.

2.5.3 Heparin Immobilization to Diaminoalkane Derivatized Cellulose Sheets

Heparin immobilization to diaminoalkane derivatized cellulose sheets was conducted analogously to that described in Section 2.5.2. Known surface areas of diaminobutane and diaminododecane derivatized cellulose sheets were reacted with tritium labeled N-acetylated heparin exactly according to the procedures described in Section 2.4.1 One hundred milliliters of carboxylic activated tritium labeled N-acetylated heparin, specific activity equaling 205,163 $\pm$ 807 dpm/mg, was added to known surface areas of each diaminoalkane derivatized cellulose sheet (∿ 25 cm^2) which were allowed to react as described in Section 2.5.2. These tritium

labeled heparin immobilization reactions were conducted in parallel with the cold heparin coupling reaction using the same batch of diaminoalkane derivatized cellulose sheets.

After repeated washings with pH 7.4 phosphate buffered saline, the tritium labeled, immobilized heparin samples were first vacuum dried and then dissolved in sulfuric acid followed by ethanol, as described in Section 2.3.4. The amount of heparin immobilized to the diaminoalkane derivatized gels was then determined by liquid scintillation counting of the above solutions.

2.5.4 Heparin Immobilization to Sepharose 4-B Beads Via Divinylsulfone

The quantity of heparin immobilized via divinylsulfone to Sepharose 4-B was determined by the difference between the amount of tritium labeled N-acetylated heparin, specific activity 43,114 dpm/mg, initially added and the summation of the total amounts of heparin present in the sequential 50 ml rinse solutions, see Section 2.4.4.

2.6 Conductimetric Titrations of Sulfate, Sulfamate and Carboxylic Groups on Functional Group Derivatized Heparins

Conductimetric titrations of heparin (95) provide a convenient quantitation of sulfate and carboxylic groups. Sodium heparin is first converted into the acid form by elution of a known quantity of heparin, dissolved in neutral deionized distilled water, through a column of previously washed AG 50W-X2 cationic

exchange resin (BioRad Laboratories). The effluent is collected until only neutral water exists in the column and is then diluted to a final volume of 150 ml.

The solution is titrated with standardized NaOH while measuring solution conductivity with a protable conductivity meter (Markson Science, Inc.) vs volume NaOH solution added. The conductance of the sample solution, initially high due mainly to the contribution of mobile protons on the $-SO_3H$ groups (specific conductance $\lambda_+ = 350$), decreased linearly as $-SO_3H$ protons are neutralized by hydroxide ions and are replaced by Na^+ ions ($\lambda_+ = 50$). After all of the $-SO_3H$ protons are neutralized, the curve levels off. This plateau region corresponds to carboxylic group proton dissociation. Conductance barely changes in this region due to the compensatory effects from carboxylic proton neutralization and increased Na^+ ion concentrations. After dissociation and neutralization of all carboxylic protons, conductance sharply increased primarily due to OH^- ion contributions ($\lambda_- = 198$). Extrapolation of the three branches of the conductimetric curves gives two intersection points, the first corresponding to the number of $-SO_3H$ groups while the second corresponds to the number of -COOH groups. Changes in the number of titratable carboxylic groups on derivatized heparins, relative to the N-acetylated heparin starting material, provides a convenient measure of the degree of carboxylic derivatization.

2.7 Proton Nuclear Magnetic Resonance Spectroscopy of Heparin and Functional Group Derivatized Heparins

Forty milligrams per ml solutions of sodium heparin and functional group derivatized sodium heparins in deuterium oxide (Stohler Isotopes) with a 2,2,-dimethyl-2-silapentane-5-sulfonate (DDS) reference compound were evaluated. Proton nmr spectra were obtained with a Varian SC 300 nmr spectrometer at ambient room temperature at the University of Utah nmr center.

2.8 Transmission Mode Infrared Spectroscopy of Cellulose Sheets

Transmission mode IR spectra of underivatized cellulose sheets, diaminoalkane derivatized cellulose sheets and heparin immobilized diaminoalkane cellulose sheets were obtained with a Beckman 620 MX IR spectrometer. Transmission IR spectra were also obtained for sodium heparin and functional group derivatized sodium heparin with KBr pellets.

2.9 X-Ray Photoelectron Spectroscopy Surface Evaluations

X-ray photoelectron spectroscopy (XPS) was conducted on cellulose, diaminoalkane derivatized cellulose, heparin immobilized diaminoalkane derivatized cellulose, and on the immobilized heparin surfaces after contact with protein solutions and plasma. XPS spectra were also obtained on N-acetylated heparin powder. All XPS polymer spectra were obtained only for flat surfaces (bead geometries were not evaluated) with a Hewlett-Packard model

5950B XPS spectrometer at the University of Utah Surface Analysis Laboratory.

2.10 In Vitro Coagulation Assays

2.10.1 Functional Group Derivatized Heparin

The anticoagulant activities of the N-acetylated heparin, the hydroxyl group derivatized heparins and the carboxylic group derivatized heparins were determined by activated partial thromboplastin time (APTT) assay methods (96). Bovine blood was collected in 3.8% sodium citrate, 9 parts blood to 1 part citrate, and centrifuged at 5000 g for 15 minutes. The supernatant plasma was collected and pooled for subsequent APTT testing. Plasma was stored at 2-4^{o}C for no longer than six hours, at which time it was discarded.

Activated partial thromboplastin time tests were conducted by first incubating 100 μl of plasma, either plasma containing known unit activities of heparin for preparation of calibration curves or plasma containing known weight concentrations of derivatized heparin for determining derivatized heparin activity, with 100 μl of activated thromboplastin reagent (Ortho Pharmaceutics) at 37^{o}C for five minutes. One hundred microliters of 0.3 M $CaCl_2$ in distilled water is then rapidly added to the incubated plasma and clotting times (APTT) were determined with a Fibrometer (Beckman). An APTT (seconds) vs heparin activity in the plasma (units/ml) is first prepared with plasma heparinized in 0.1 unit/ml increments.

A linear relationship is typically observed between 0.0 and 0.5 units/ml of heparin; if r, linear regression correlation coefficient, is less than 0.90 the plasma was discarded without further testing. After preparation of the APTT vs plasma heparin activity curve, known weight concentrations of the functional group derivatized heparins were prepared with the same plasma used to obtain the APTT calibration curve and APTT was determined as with the standard heparin activity calibration plasmas. Unheparinized plasma and selected standard heparin calibration plasmas were periodically evaluated to ensure that coagulation factors had not degraded during the course of testing. If control or standard heparin activity plasmas deviated from original values, the testing was discontinued and that plasma was discarded. Each plasma sample was tested for APTT in triplicate for each batch of plasma and derivatized heparin activities were determined based on a minimum of 6 batches of plasma collected from different animals at different times.

Having previously prepared the APTT plasma heparin activity curve and determined APTT for known heparin plasma concentrations (mg/ml), the plasma heparin activity (units/ml) is determined from the calibration curve and the derivatized heparin activity is then determined (units/mg)

2.10.2 Immobilized Heparin Gels

Activated partial thromboplastin times were also conducted on plasmas exposed to immobilized heparin gels to determine spacer

arm length effects on immobilized heparin anticoagulant activity and possible differences in anticoagulant activity associated with immobilization via different heparin functional groups. The APTT vs plasma heparin activity curve was prepared as described above in Section 2.10.1. Various quantities of the heparinized gels were then added to 10 ml of the same plasma used to prepare the calibration curve. The gels and plasmas were then gently rotated at approximately 1 rps for 10 minutes at room temperature and then centrifuged at 2500 g for 5 minutes, APTT was then determined for the supernatant plasmas. Activated partial thromboplastin times were also determined for the diaminoalkane derivatized gels without immobilized heparin, in the same gel volume to plasma volume ratio. Free heparin levels in gel exposed plasma samples were determined by liquid scintillation counting of the tritium labeled heparin, specific activity 43,114 dpm/mg.

2.11 Protein Adsorption onto Heparinized Diaminoalkane Derivatized Cellulose Sheets

2.11.1 Protein Adsorption from Single Component Protein Solutions

Ten milligrams per ml solutions of fraction V bovine albumin, essentially fatty acid free (Sigma Chemical Company), purified human antithrombin III (American Red Cross) and purified bovine fibrinogen (IMCO) in pH 7.4 phosphate buffered saline were prepared. The pH's of the albumin, ATIII and fibrinogen solutions

were 7.2, 7.3 and 7.3 representively. Portions of the dessicated heparinized diaminoalkane derivatized cellulose sheets ($\sim$ 2-5 cm^2) were first hydrated for 30 minutes in pH 7.4 phosphate buffered saline, dried with lens cleaning paper and then placed in the above solutions for 2 hours at room temperature. The heparinized cellulose samples were then removed from the protein solutions and placed in 20 ml of PBS and rotated at 1 rps for 10 minutes. The samples were then removed and placed in 20 ml of fresh PBS and rotated an additional 10 minutes at which time the samples were vaccum dried and stored under vacuum dessication for XPS surface analysis.

2.11.2 Protein Adsorption from Bovine Plasma

Portions of the heparinized diaminoalkane derivatized cellulose sheets ($\sim$ 2-5 cm^2) were hydrated in pH 7.4 phosphate buffer saline for 30 minutes, dried with lens cleaning paper and then placed in bovine plasma, prepared from calf's blood freshly collected in Na citrate, for 2 hours at room temperature. The heparinized diaminoalkane derivatized cellulose samples were then rinsed as described in Section 2.11.1 above, vacuum dried and stored under vacuum dessication for subsequent XPS analysis.

2.12 Platelet Retention and Platelet Factor 4 Release for Heparin Immobilized Diaminoalkane Derivatized Sepharose 6-MB Beads

Platelet retention and α-granule release were determined by

passing whole blood through columns containing respective gels and determining platelet levels and plasma PF4 levels in fractions collected existing the columns. Polypropylene columns were packed with 1 cc of each respective diaminoalkane gel (without heparin) and each diaminoalkane spacer arm mediated heparin immobilized gel. Control materials consisted of untreated Sepharose 6-MB gel. Twenty-five milliliters of calf's blood, freshly collected via a double syringe technique in citrate, was perfused through each column at a controlled flow rate of 2.0 cc/min with a peristaltic pump, and 5.0 cc whole blood fractions were sequentially collected exiting each column. One milliliter of whole blood was removed from each fraction for hematocrit and platelet level determination using a Coulter Counter(R). The remaining portion of each blood fraction was immediately cooled in an ice water bath for between 30 minutes and 2 hours. After 25 cc of whole blood perfusion, 15 cc of PBS was perfused through each column to rinse the gels, followed by 15 cc of 1.0 M NaCl to elute PF4 bound to immobilized heparin (97). These fractions were collected and placed in the ice water bath as previously described. Platelet poor plasma was then obtained from the cooled blood fractions by centrifugation. These plasma samples were immediately frozen (-20^{o}C) for later PF4 analysis. The PBS and 1.0 M NaCl fractions were similarly centrifuged and the resultant supernatants were collected and frozen for PF4 analysis. Each material was ran in triplicate using different calves.

Platelet factor 4 levels in collected plasma and buffer frac-

tions were determined by bovine PF4 radioimmunoassay techniques using bovine PF4 RIA reagents (Abbott Laboratories). The test is a competitive radioimmunoassay in which nonradioactive PF4 in plasma, and buffer, competes with a constant amount of ^{125}I-PF4 for binding sites on a constant limited amount of bovine PF4 antiserum. The percentage of radioactive PF4 bound to the antiserum is therefore inversely proportional to the concentration of nonlabeled PF4 in the test samples. To 250 µl of the ^{125}I-PF4 solution is added either 50 µl of dilution buffer, nonlabeled bovine PF4 standards in concentrations ranging from 50 ng/ml to 1000 ng/ml, or 50 µl of the test sample. Two hundred and fifty microliters of PF4 antiserum is added and the tubes are incubated at room temperature for 2 hours after which 1 ml of 73% saturated ammonium sulfate is added. The tubes are then incubated between 10 and 60 minutes and then centrifuged at 1500 g for 20 minutes. The supernatant is carefully decanted into liquid waste and the excess supernatant on the rim of each tube is blotted with tissue paper. The total Iodine-125 in the precipitate is then counted with a gamma counter. The percent bound, expressed as cpm of standard or test sample divided by the total cpm in 250 µl of the ^{125}I-PF4 solution multiplied by 100, is determined for each tube. A standard curve is then prepared by plotting percent bound vs standard PF4 concentration. By locating percent bound of the unknown test sample on the standard curve, the concentration of PF4 in the test sample is determined. Each PF4 standard and unknown test sample, plasma or buffer solution, is evaluated in triplicate.

CHAPTER 3

RESULTS

3.1 Diaminoalkane Coupling to Polymer Substrates

3.1.1 Diaminoalkane - Agaroses

The degrees of diaminoalkane coupling to respective agarose gels was determined by the manufacturer and are presented in Table IV.

3.1.2 Diaminoalkane Coupling to Sepharose 6-MB

The degrees of diaminoalkane coupling to Sepharose 6-MB beads, to be used in platelet retention and PF4 release experiments, were determined by a carbodiimide reaction with an excess of carbodiimide and ^{14}C-acetic acid. After thorough washing in pH 7.4 phosphate buffered saline, known volumes of ^{14}C-acetate coupled diaminobutane and diaminododecane derivatized Sepharose 6-MB gels were digested in concentrated acid, diluted with ethanol and counted for carbon-14. Quenching was determined for each sample by H-number methods on a Beckman LS-7500 liquid scintillation counter. Based on blank scintillation fluid vials, a carbon-14 background of 15.0 cpm was subtracted from the total counts of each sample and corrected dpm, calculated based on origin counting efficiencies, were used in subsequent calculations. The moles of

Table IV

Diaminoalkane Coupling to
Agarose Gels

Diaminoalkane-Agarose	Degree of Coupling ($\frac{\mu \text{ moles}}{\text{cc gel}}$)
1,2-diaminoethane-agarose	4.6
1,4-diaminobutane-agarose	8.8
1,8-diaminooctane-agarose	2.5
1,10-diaminodecane-agarose	4.8
1,12-diaminododecane-agarose	2.7

^{14}C-acetate coupled per cubic centimeter of gel plus or minus one standard deviation, based on three assays, is presented in Table V below. These values were taken as the amount of accessible free primary amines, coupled diaminoalkanes with a free primary amine, capable of covalent coupling with heparin.

3.1.3 Diaminoalkane Coupling to Cyanogen Bromide Activated Cellulose Sheets

The degrees of diaminoalkane coupling to the cyanogen bromide activated cellulose sheets were determined by a carbodiimide reaction with an excess of carbodiimide and ^{14}C-acetate acid. After thorough washing with pH 7.4 phosphate buffered saline, known surface areas of the ^{14}C-acetate coupled diaminobutane and diaminododecane derivatized cellulose sheets were digested in concentrated acid, diluted with ethanol and counted for carbon-14. Quenching was determined for each sample by H-number methods and a carbon-14 background of 15.0 cpm was substracted from each sample. Total dpms were then recalculated based on the corrected cpm values and these background corrected dpm values were used in subsequent calculations.

The moles of ^{14}C-acetate coupled per square centimeter of cellulose plus or minus one standard deviation, based on three assays, are presented in Table VI below. These values were taken as the amount of accessible free primary amines, coupled diaminoalkanes with a free primary amine, capable of covalent coupling with heparin.

Table V

Diaminoalkane Coupling to
Sepharose 6-MB

Diaminoalkane-Sepharose	Degree of Coupling ($\frac{\mu \text{ moles}}{\text{cc gel}}$)
1,4-diaminobutane-Sepharose	2.29 $\pm$ 0.31
1,12-diaminododecane-Sepharose	2.05 $\pm$ 0.12

Table VI

Diaminoalkane Coupling to
Cyanogen Bromide Activated
Cellulose Sheets

Diaminoalkane-Cellulose	Degree of Coupling ($\frac{\mu \text{ moles}}{\text{cm}^2}$)
1,4-diaminobutane-cellulose	0.048 $\pm$ 0.003
1,12-diaminododecane-cellulose	0.052 $\pm$ 0.005

3.2 Heparin Coupling to Polymer Substrates

3.2.1 Heparin Coupling to Diaminoalkane-Agaroses

The amount of heparin coupled to the respective diaminoalkane-agarose gels was determined by the difference between the total amount of heparin used in the immobilization reactions, specific activity 43,114 dpm/mg, and the total amount of heparin collected in subsequent gel washings. For convenience sake, the washings were pooled and tritium levels were determined on the pooled washings having a total final volume of 500 ml for each gel, no heparin was detected in fraction's after the tenth fraction. Quenching was determined for each sample by H-number methods using a Beckman LS-9000 liquid scintillation counter and tritium background levels were substracted from each sample. The amount of tritium labeled heparin, specific activity 43,114 dpm/mg, coupled to each diaminoalkane gel is presented in Table VII below. These heparin immobilized gels were then used in subsequent clotting time tests.

3.2.2 Heparin Coupling to Diaminoalkane Derivatized Sepharose 6-MB

The degrees of heparin coupling to diaminobutane and diaminododecane derivatized Sepharose 6-MB beads was determined for an exact parallel reaction with tritium labeled heparin, specific activity 205,163 $\pm$ 807 dpm/mg. After thorough washing, known volumes of gels were digested in concentrated acid, diluted with

Table VII

Heparin Immobilization to
Diaminoalkane-Agarose

Diaminoalkane-Agarose	Degree of Heparin Immobilization ($\frac{mg}{cc\ swollen\ gel}$)
1,2-diaminoethane-agarose	0.50
1,4-diaminobutane-agarose	0.97
1,8-diaminooctane-agarose	3.14
1,10-diaminodecane-agarose	2.33
1,12-diaminododecane-agarose	0.95

ethanol and counted for tritium levels. Quenching was determined for each smaple by H-number methods using a Beckman LS-7500 liquid scintillation counter. Based on control scintillation fluid samples a tritium background level of 24.5 cpm was subtracted from each sample and the corrected dpm was determined based on the original counting efficiency, assays were conducted in triplicate. The values obtained for heparin coupling with the tritium labeled heparin plus or minus one standard deviation, were assumed to be equal to the degree of coupling for the nonlabeled heparin coupling reactions and are presented in Table VIII below.

3.2.3 Heparin Coupling to Diaminoalkane Derivatized Cellulose

The degrees of heparin coupling to diaminobutane and diaminododecane derivatized cellulose sheets was determined for an exact parallel reaction using tritium labeled heparin, specific activity 205,163 $\pm$ 807 dpm/mg. After thorough washing, known surface areas of the immobilized heparin materials were digested in concentrated acid, diluted with ethanol and counted for tritium levels. Quenching was determined for each sample and background tritium levels were subtracted for each sample as described in Section 3.2.2. The values obtained for heparin coupling with tritium labeled heparin plus or minus one standard deviation were assumed to equal the degrees of coupling for the nonlabeled heparin coupling reactions and are presented in Table IX.

Table VIII

Heparin Coupling to Diaminoalkane
Derivatized Sepharose
6-MB Beads

Diaminoalkane-Sepharose	Degree of Heparin Immobilization ($\frac{mg}{cc\ swollen\ gel}$)
1,4-diaminobutane-Sepharose	1.46 ± 0.07
1,12-diaminododecane-Sepharose	1.48 ± 0.09

Table IX

Heparin Coupling to Diaminoalkane
Derivatized Cellulose

Diaminoalkane-Cellulose	Degree of Heparin Immobilization ($\frac{\mu g}{cm^2}$)
1,4-diaminobutane-cellulose	6.06 ± 0.98
1,12-diaminododecane-cellulose	5.18 ± 0.57

3.2.4 Heparin Coupling to Sepharose 4-B Via Divinylsulfone

As in Section 3.2.1, the degree of heparin coupling via divinylsulfone to Sepharose 4-B beads was determined by the difference between the total amount of heparin, specific activity 43,114 dpm/mg, used in the immobilization reaction and the total amount of heparin collected in subsequent washings. For convenience sake, the washings were pooled for the first 10 wash fractions and tritium levels were determined for the pooled washings, a total final volume of 500 ml. The amount of tritium labeled heparin immobilized via a divinylsulfone bridge was determined to be 2.05 mg/cc gel.

3.3 Conductimetric Titrations of Functional Group Derivatized Heparins

3.3.1 Conductimetric Titrations of N-acetylated Heparin

Conductimetric titration of N-acetylated heparin were conducted in triplicate. The total amounts of carboxylic and sulfate (both sulfate and sulfamate) groups per mg of N-acetylated heparin, plus or minus one standard deviation, were determined to be $1.68 \times 10^{-6} \pm 0.08 \times 10^{-6}$ mole $-COO^-$/mg and $3.05 \times 10^{-6} \pm 0.18 \times 10^{-6}$ mole $-SO_3^-$/mg respectively. A representative conductimetric titration curve for N-acetylated heparin is presented in Figure 9.

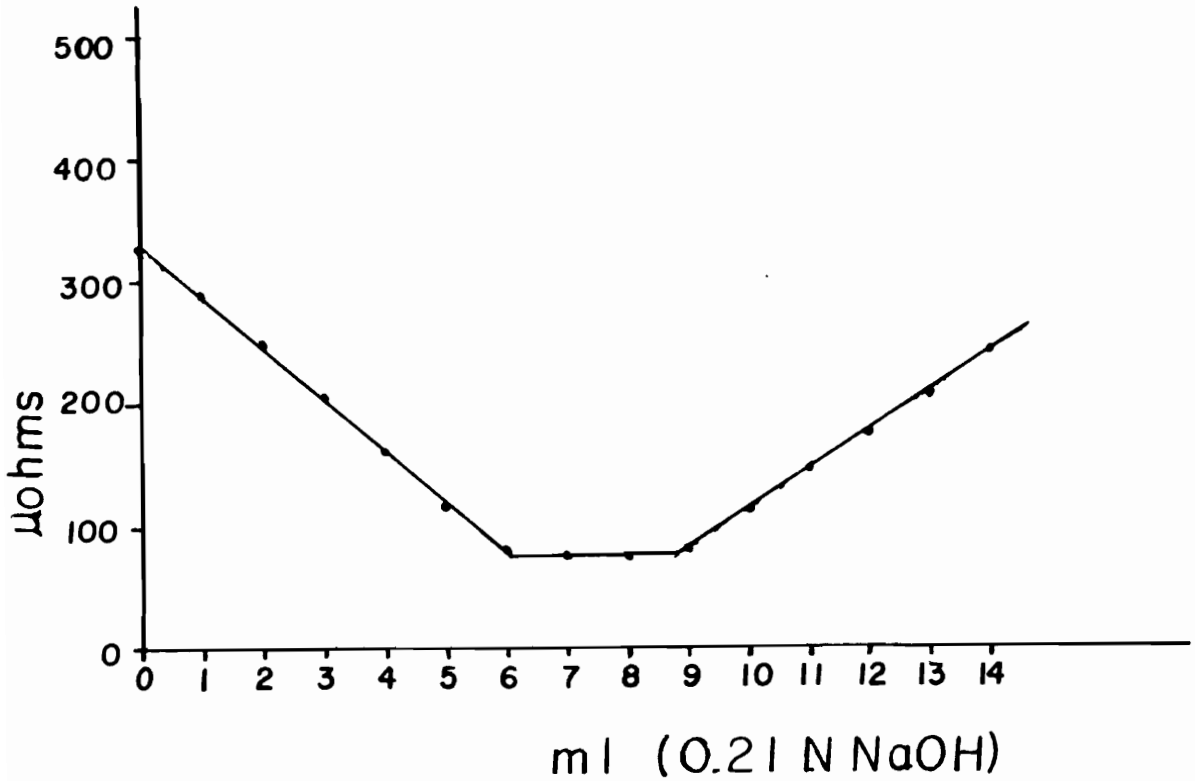


Figure 9. Conductimetric titration curve for N-acetylated heparin.

3.3.2 Conductimetric Titrations of Carboxylic Derivatized Heparin

The effects of the degree of carboxylic blocking with n-butylamine on N-acetylated heparin were determined by sampling portions of the carbodiimide reaction with time, sampling times were at 1 hour and 20 minutes, 4 hours and 6 hours. Carboxylic groups on N-acetylated heparin were also blocked with 2-aminoethyl hydrogensulfate via the carbodiimide reaction. Total carboxylic groups and sulfate groups, both sulfate and sulfamate, were determined based on conductimetric titrations with standardized NaOH solutions for the carboxylic blocked N-acetylated heparin and are presented in Table X. Conductimetric titration curves for the 1 hour and 20 minutes and the 6 hour n-butylamine samples are presented in Figures 10 and 11.

The conductimetric titration data provides a convenient means for approximately the degree of carboxylic group blocking. Based on an initial carboxylic group abundance of 1.68 μ moles/mg, the percent blockage of the carboxylic groups are 17.9%, 36.9% and 100% for the n-butylamine reaction at 1 hour plus 20 minutes, 4 hours and 6 hours reaction time respectively. Similarily the degree of carboxylic blocking for the 2-aminoethyl hydrogensulfate reaction is 10.1%. The relative abundance of sulfate groups progressively decreases with time, either the result of increased molecular weight associated with derivatization or the results of acid catalyzed hydrolysis of sulfate and sulfamate groups. Undoubtedly, the relative sulfate content will decreased with in-

Table X

Carboxylic and Sulfate Groups Determinations for Carboxylic Blocked N-acetylated Heparin

Ligand	COOH ($\frac{\mu \text{ moles}}{\text{mg}}$)	$-SO_3H$ ($\frac{\mu \text{ moles}}{\text{mg}}$)
n-butylamine (1 hr 20 min reaction time)	1.38	3.08
n-butylamine (4 hr reaction time)	1.06	2.87
n-butylamine (6 hr reaction time)	0	2.66
2-aminoethyl hydrogen sulfate	1.51	3.76

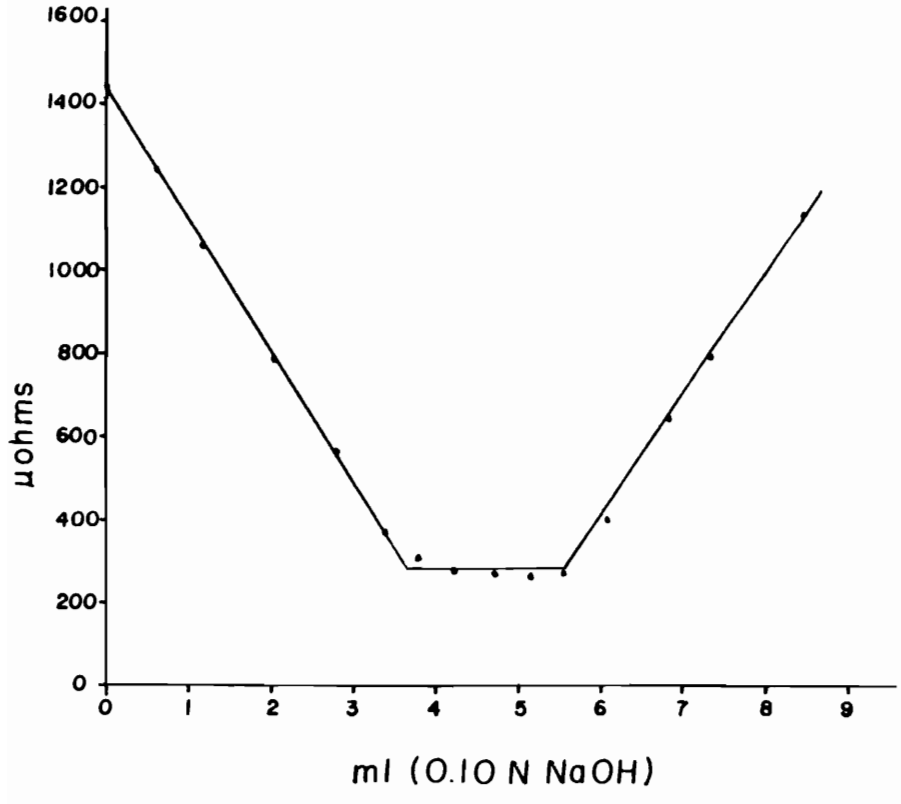


Figure 10. Conductimetric titration curve for 1 hour and 20 min. reaction time carboxylic blocked heparin.

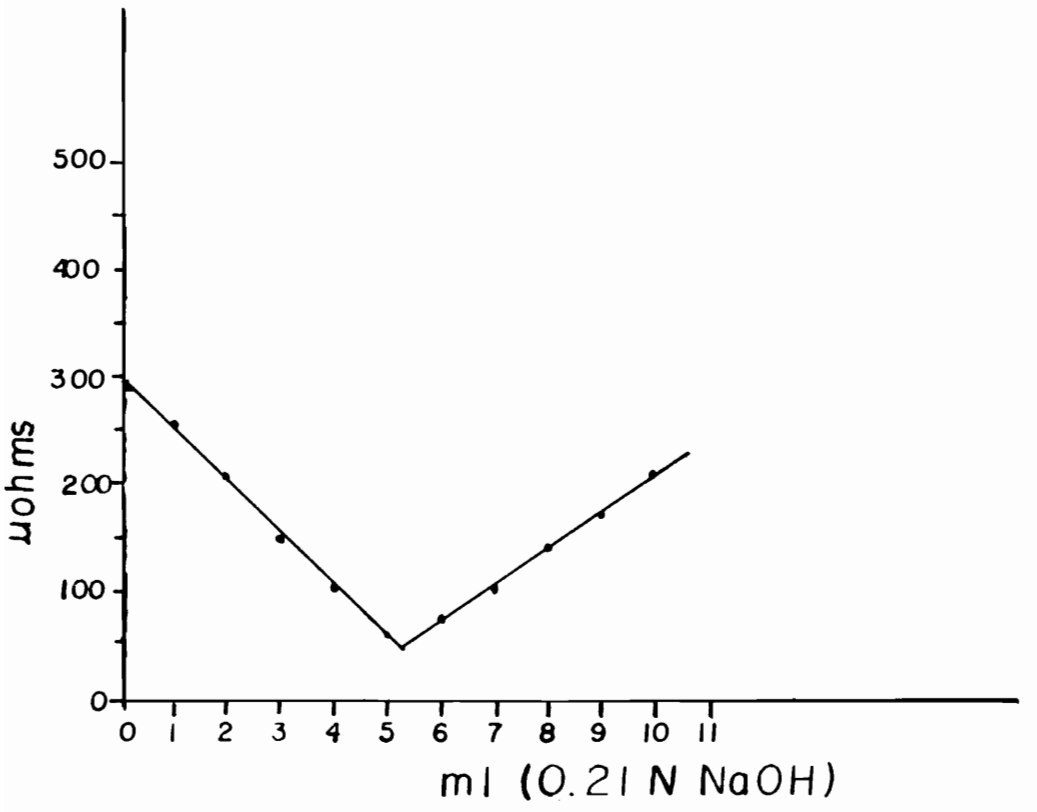


Figure 11. Conductimetric titration curve for 6 hour reaction time carboxylic blocked heparin.

creased derivatization and resultant increased molecular weight; however, the loss of sulfates from hydrolysis at pH 4.75 cannot be ruled out based on this data.

3.3.3 Conductimetric Titrations of Hydroxyl Derivatized N-acetylated Heparin

3.3.3.1 Epichlorohydrin Reactions

Hydroxyl groups on N-acetylated heparin were blocked with n-butylamine, 2-aminoethyl hydrogensulfate and glycine using the crosslinking agent epichlorohydrin. Total carboxylic and sulfate groups on hydroxyl derivatized N-acetylated heparin were determined by conductimetric titrations and are listed in Table XI.

It is difficult to determine the degrees of hydroxyl group derivatization based on the data presented here. As before, one expects the relative abundance of carboxylic and sulfate groups to decrease with increased derivatization, with the n-butylamine and this is found to be the case. This task is further complicated by the high polydispersity, both molecular weight and chemical structure, of heparin along with the fact that epichlorohydrin is a crosslinking agent. Although primary amines on the heparin have been blocked, crosslinking between hydroxyl groups on heparin molecules cannot be ruled out.

3.3.3.2 Divinylsulfone Reactions

Hydroxyl groups on N-acetylated hpearin were blocked with n-

Table XI

Carboxylic and Sulfate Group Determinations
for Hydroxyl Derivatized N-acetylated
Heparin-Epichlorohydrin Reactions

Ligand	-COOH ($\frac{\mu \text{ moles}}{\text{mg}}$)	$-SO_3H$ ($\frac{\mu \text{ moles}}{\text{mg}}$)
n-butylamine	1.29	2.20
2-aminoethyl hydrogensulfate	1.33	2.86
glycine	1.71	3.05

butylamine, 2-aminoethyl hydrogensulfate and glycine using divinylsulfone as the crosslinking agent. Total carboxylic and sulfate on the hydroxyl derivatized n-acetylated heparins were determined by conductimetric titrations and are listed in Table XII.

The problems associated with interpreting the conductimetric titration data, in regards to determining the degree of derivatization, discussed for the epichlorohydrin reactions applies equally, if not more so, with the divinylsulfone reactions. Certainly crosslinking is expected between hydroxyl groups on heparin molecules with divinylsulfone.

3.4 Proton Nuclear Magnetic Resonance Spectroscopy of Heparin

The proton nmr spectra for untreated sodium heparin, literally "out of the bottle," and N-acetylated sodium heparin are shown in Figures 12 and 13 respectively. Broad carbon proton peaks are observed between $\sim$ 2.6 and $\sim$ 4.8 ppm with a large solvent peak at 3.96 ppm. A triplet peak is observed at 0.98 ppm and an apparent quartet is also observed at 3.04 ppm for the untreated heparin. After acetylation and dialysis, the broad carbon proton peaks and the solvent peak appear unchanged; however, the triplet at 0.98 ppm and the quadruplet at 3.04 ppm are no longer present. These two peaks must represent impurities in the original heparin source that are removed by dialysis. These impurity peaks might arise from ethanol used to precipitate the sodium heparin during the commercial preparation of the anticoagulant.

Table XII

Carboxylic and Sulfate Group Determinations
for Hydroxyl Derivatized N-acetylated
Heparin-Divinylsulfone Reaction

Ligand	-COOH ($\frac{\mu \text{ moles}}{\text{mg}}$)	$-SO_3H$ ($\frac{\mu \text{ moles}}{\text{mg}}$)
n-butylamine	1.48	2.70
2-aminoethyl hydrogensulfate	1.30	3.01
glycine	1.21	2.57

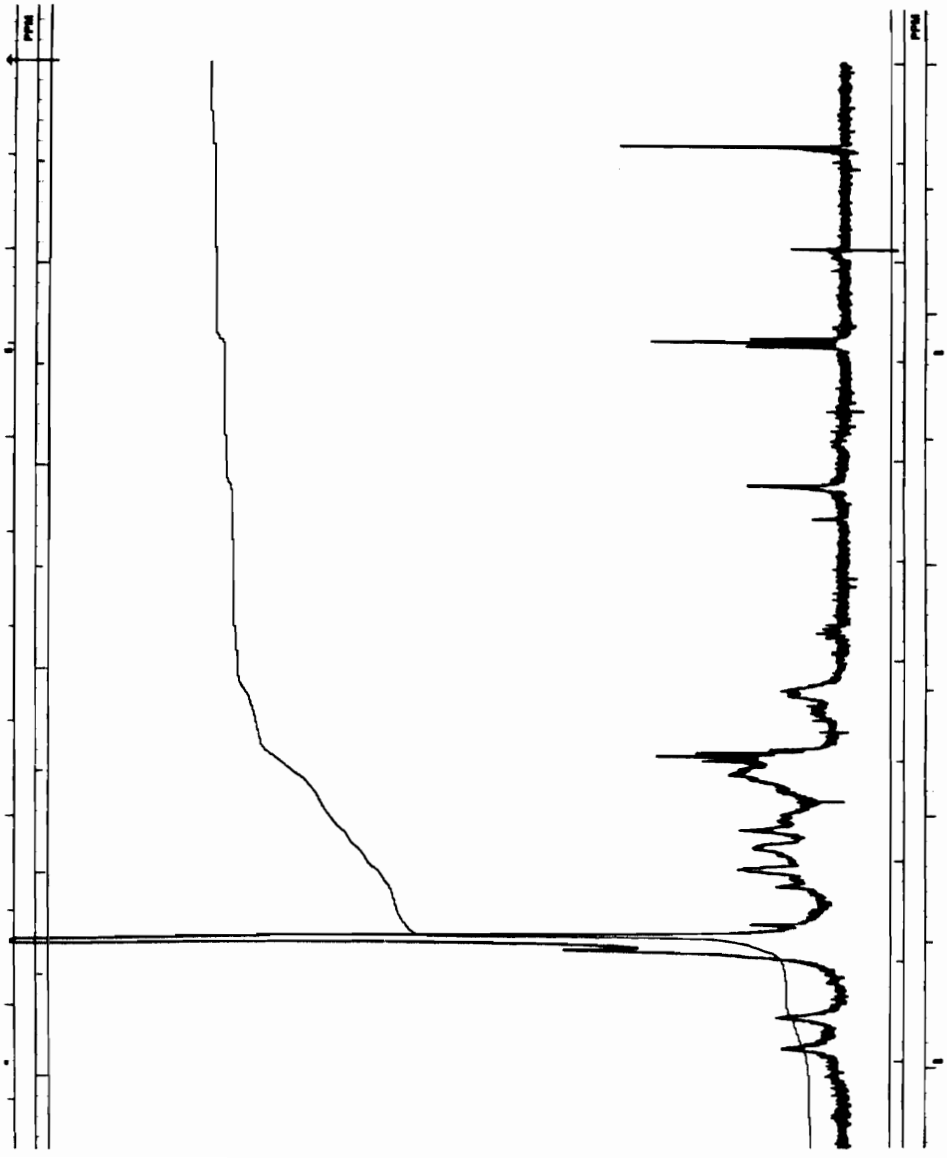


Figure 12. Proton nmr spectrum for untreated heparin.

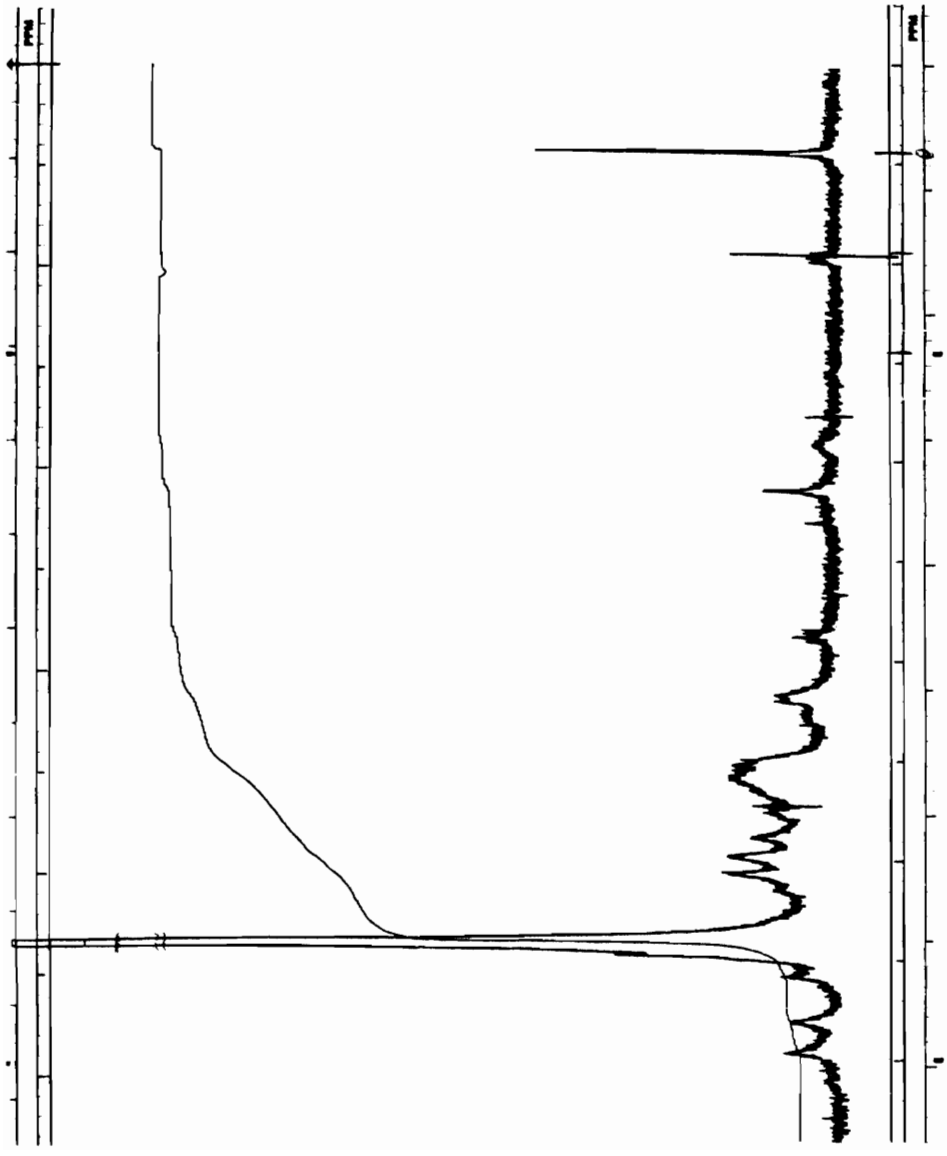


Figure 13. Proton nmr spectrum for N-acetylated heparin.

3.5 Infrared Spectroscopy

3.5.1 Functional Group Derivatized Heparin

The IR spectrum, transmission mode, for N-acetylated heparin is shown in Figure 14. A broad peak between ∿ 3700 and ∿ 3000 cm^{-1} arises from hydroxyl and amine proton stretching and the characteristic carboxyl anion peak is observed at ∿ 1650 cm^{-1}. The IR spectrum for the epichlorohydrin reaction with n-butylamine is shown in Figure 15. The peak at 2960 cm^{-1}, alkane C-H stretching, is substantially larger for the n-butylamine hydroxyl derivatized N-acetylated heparin, relative to the N-acetylated heparin starting material. Other than this single peak, the IR spectrums for the functional group derivatized heparins are not significantly different from the N-acetylated heparin starting material.

3.5.2 Cellulose Sheet Derivatizations

The IR spectrum, transmission mode, for the soxhlet extracted cellulose sheets is presented in Figure 16. Characteristic groups include the broad hydroxyl stretching peak between ∿ 3700 and 3000 cm^{-1} and the carbon-hydrogen stretching peak at ∿ 2900 cm^{-1}. After cyanogen activation and diaminoalkane coupling, the carbon-hydrogen stretching peak increases and a new peak at approximately 1730 cm^{-1} appears due to the formation of the imidocarbonate group from the cyanogen bromide. The IR spectrum for diaminododecane coupling to the cellulose sheet is presented in Figure 17 as a representative spectrum. After heparin coupling, the car-

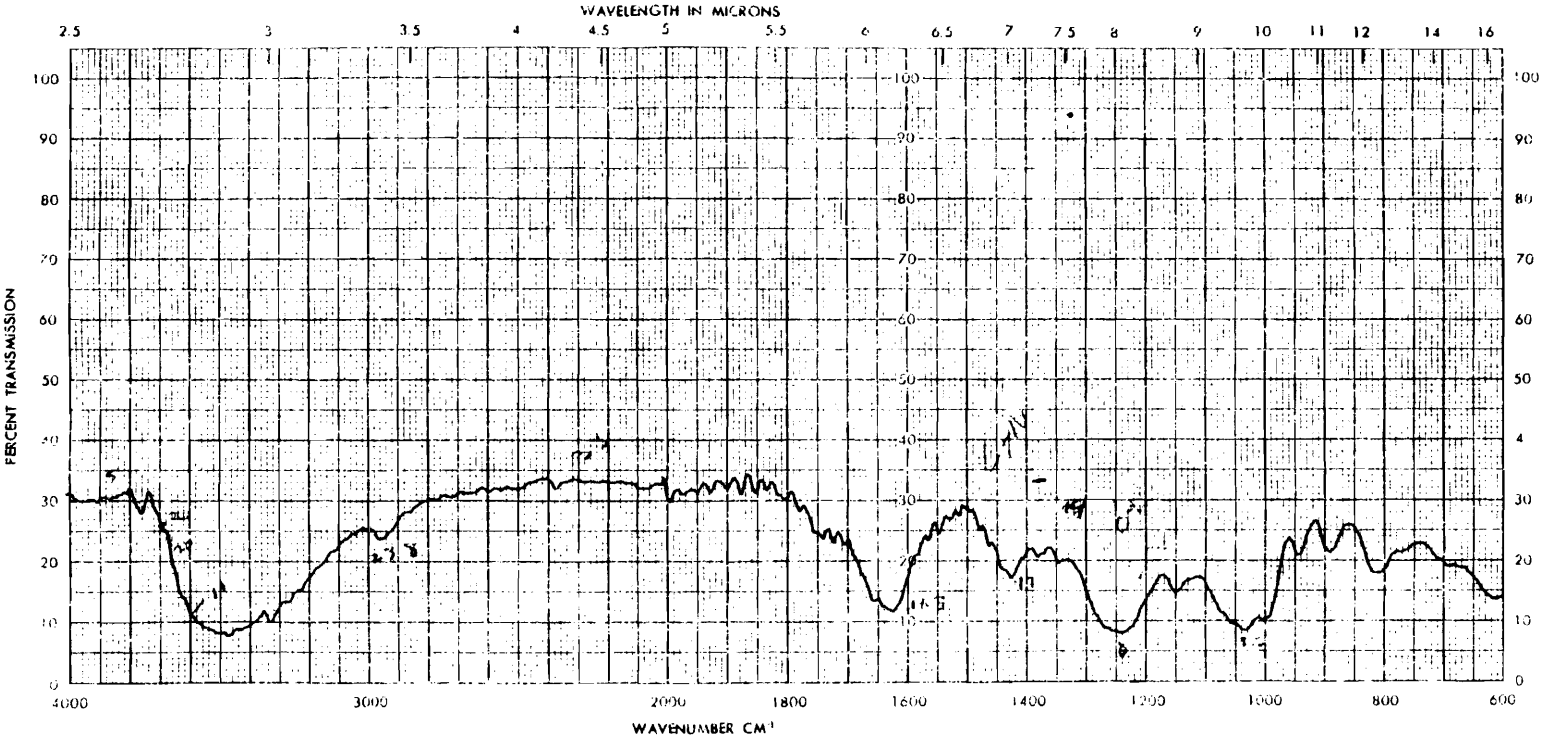


Figure 14. Infrared spectrum for N-acetylated heparin.

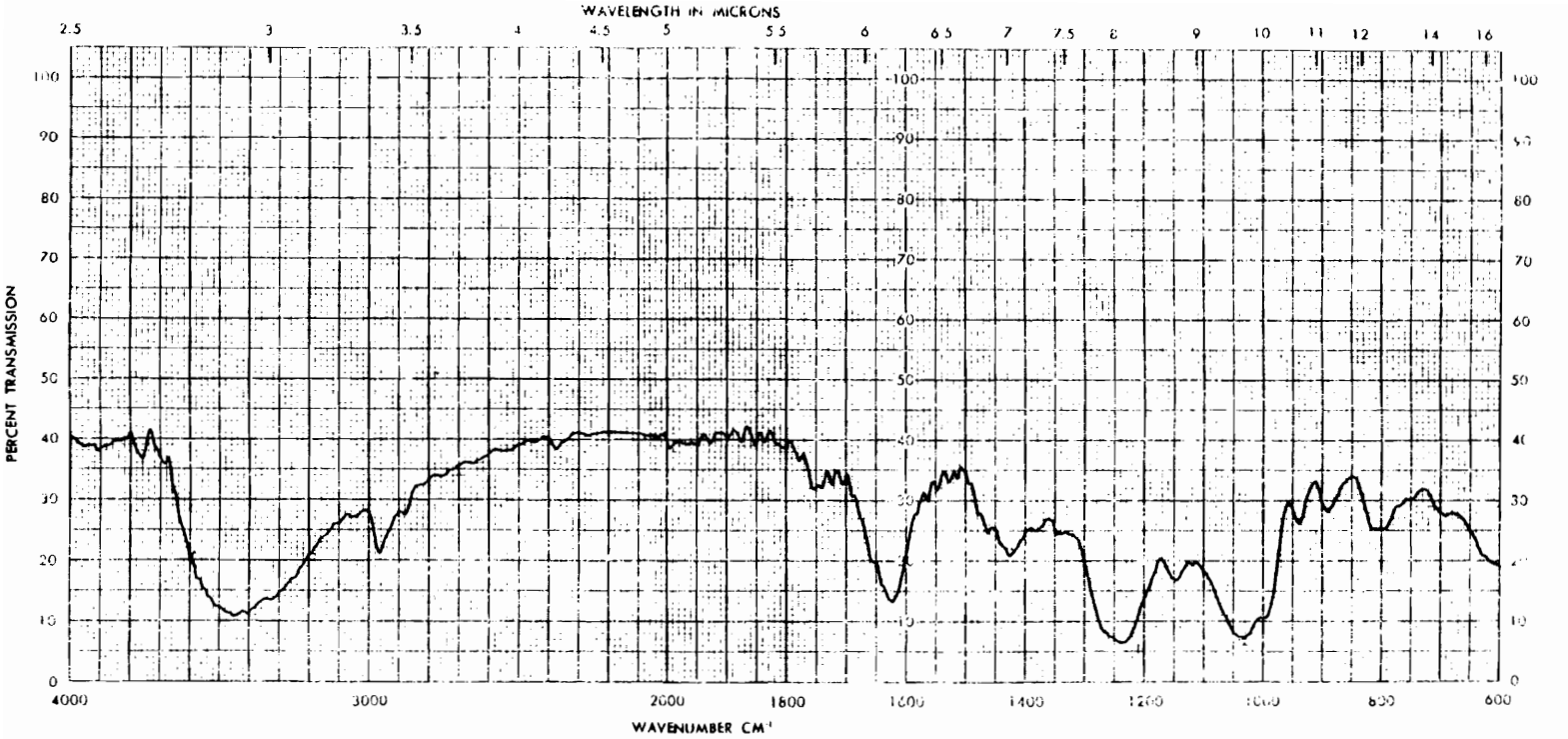


Figure 15. Infrared spectrum for n-butylamine hydroxyl derivatized heparin.

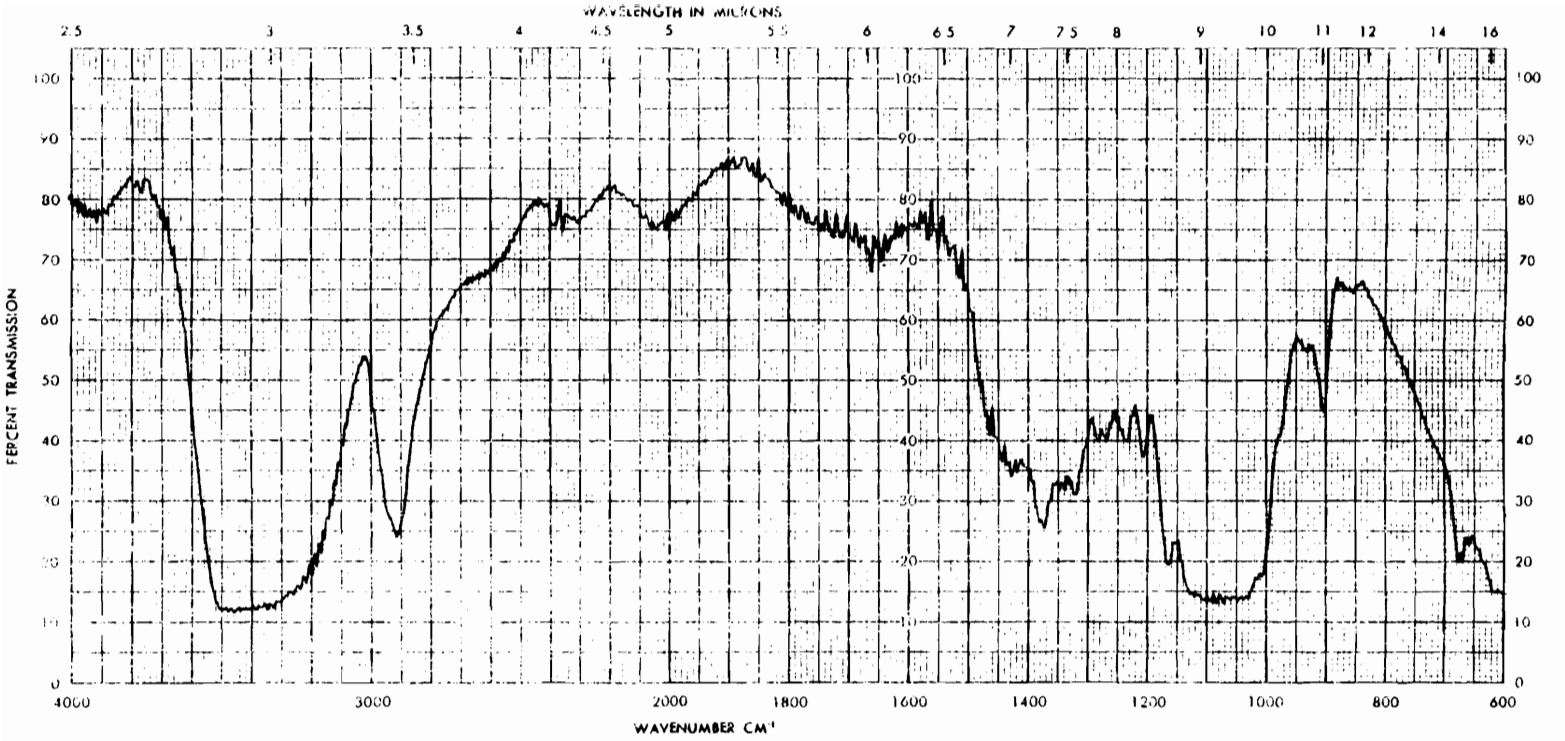


Figure 16. Infrared spectrum for cellulose sheets.

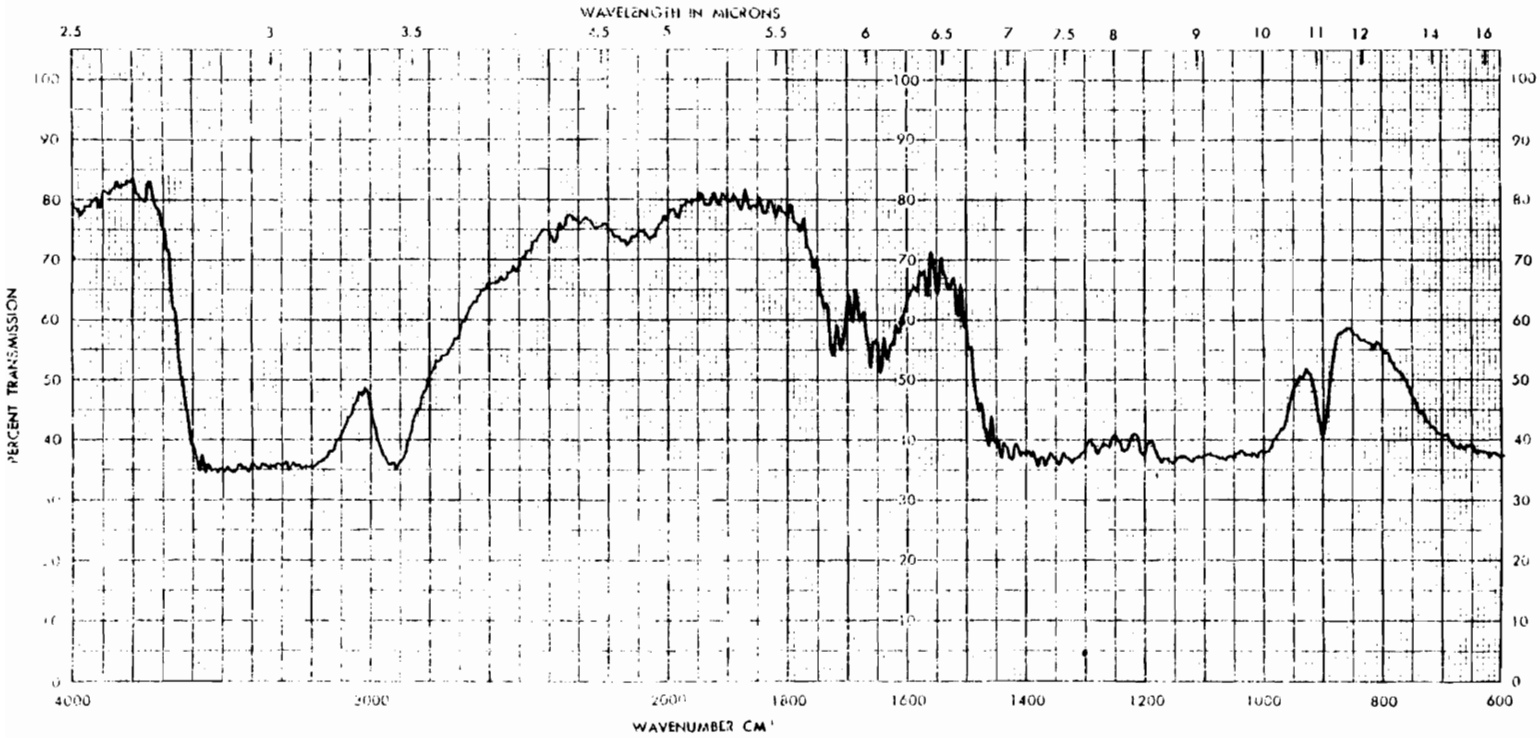


Figure 17. Infrared spectrums for diaminododecane derivatized cellulose sheets.

boxyl anion and newly formed amide peaks, both at $\sim$ 1650 cm^{-1}, from the heparin are observed. The IR spectrum for heparin coupled to diaminododecane derivatized cellulose sheets is presented in Figure 18 as a representative spectrum.

3.6 X-Ray Photoelectron Spectroscopy

X-ray photoelectron spectroscopy was conducted on extracted cellulose, diaminobutane and diaminododecane derivatized cellulose, heparin powder and heparin immobilized to the respective diaminoalkane derivatized celluloses. Flood gun conditions were optimized for the initial cellulose material, 0.6 ma and 6.0 eV, and those settings were used in all subsequent analyses. Atomic percents for various elements were determined by the equation:

$$\text{Atomic Percent}_i = \left(\frac{\text{Peak Area}_{i/\sigma_i \cdot N_i}}{\Sigma \text{Peak Area}_{n/\sigma_n \cdot N_n}} \right) \times 100$$

where σ_i is the photoionization cross section for specific electrons of element i and N_i is the number of scans of the specific electrons for the element i.

3.6.1 XPS Analyses for Cellulose Substrates

The atomic percents of carbon, oxygen and nitrogen were determined and are listed below, including the appropirate photoionization cross sections, in Table XIII. The nitrogen $1S_{1/2}$ peak is extremely small and probably represents some form of surface contamination. This surface contamination is further brought

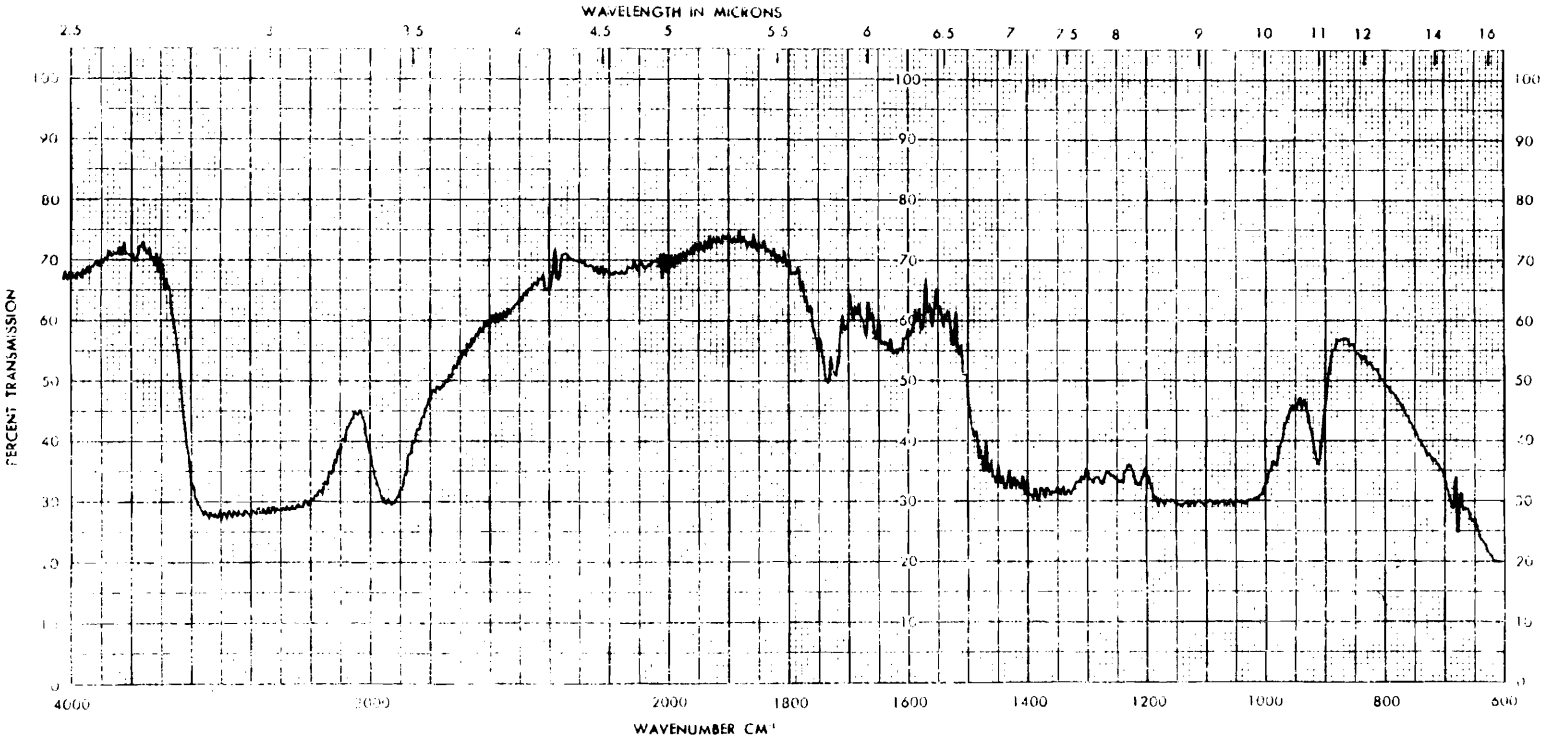


Figure 18. Infrared spectrum for heparin coupled to diaminododecane derivatized cellulose sheets.

Table XIII

Atomic Percent for
Cellulose Sheets

Elements	Cross Section-Electron Type	Number of Scans	Atomic Percent
C	1.00 ($1S_{1/2}$)	10	61.3
N	1.80 ($1S_{1/2}$)	20	0.5
O	2.93 ($1S_{1/2}$)	10	38.2

out by the high resolution spectrum of the carbon $1S_{1/2}$ electrons shown in Figure 19. Based on the carbon structure of cellulose, one would expect two types of carbons to be present, carbon atoms bound to a single oxygen and carbon atoms bound to 2 oxygens (glycosidic carbons), in 5:1 proportions respectively. However, three carbon peaks are observed: peak #1 at 289.44 eV, peak #2 at 285.60 eV and peak #3 at 284.00 eV. All peaks are corrected to the alkane carbon $1S_{1/2}$ peak which should occur at 284.00 eV, approximately 6 eV must be added to each peak. The relative peak areas for the three peaks are 12.04%, 66.48% and 21.48% respectively. Peaks #1 and #2 therefore, represent the cellulose carbon atoms and peak #3, low binding energy, must represent surface contamination. The oxygen $1S_{1/2}$ peak appears as a single component peak.

3.6.2 Diaminoalkane XPS Derivatized Cellulose Analyses

The atomic percents of carbon, oxygen and nitrogen are presented in Table XIV for the diaminoalkane derivatized cellulose sheets.

The atomic percents of nitrogen have increased for both diaminoalkane coupled cellulose sheets. These nitrogen atoms arise from the imidocarbonate group from the cyanogen bromide activation step and the amine groups from the diaminoalkanes. The fact that the atomic percent nitrogen is higher for diaminobutane than for diaminododecane can be explained based on the increased amount of

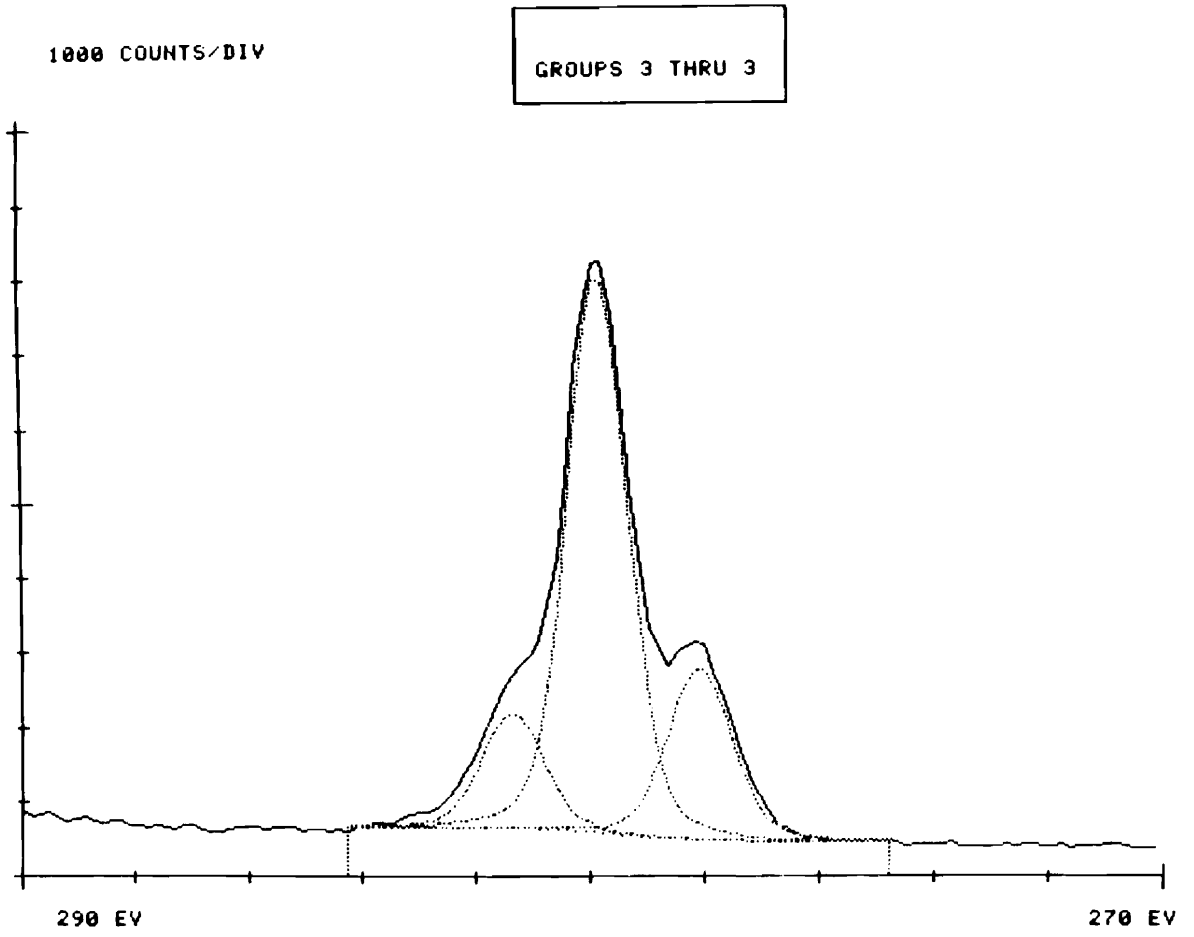


Figure 19. XPS spectrum of carbon $1S_{1/2}$ electrons for cellulose sheets.

Table XIV

Atomic Percents for Diaminoalkane
Derivatized Cellulose Sheets

Diaminoalkane	Element	Cross Section-Electron Type	Number of Scans	Atomic Percent
Diaminobutane	C	1.00-$1S_{1/2}$	10	65.2
Diaminobutane	N	1.80-$1S_{1/2}$	20	2.8
Diaminobutane	O	2.93-$1S_{1/2}$	10	32.0
Diaminododecane	C	1.00-$1S_{1/2}$	10	63.5
Diaminododecane	N	1.80-$1S_{1/2}$	20	1.8
Diaminododecane	O	2.93-$1S_{1/2}$	10	34.7

carbon associated with the diaminododecane spacer arm; however, the atomic percent of carbon for the diaminododecane derivatized cellulose was actually lower than for the diaminobutane derivatized cellulose. High resolution carbon $1S_{1/2}$ spectra for the diaminobutane and diaminododecane derivatized celluloses are presented in Figures 20 and 21, respectively. Three major carbon $1S_{1/2}$ peaks are observed with both spectrums; however, the low energy, alkane carbon portions of the total carbon spectra progressively increases with the diaminobutane and diaminododecane derivatized celluloses as would be expected. With the diaminobutane cellulose, the alkane carbon represents 33.7% percent of the total carbon while the alkane carbon peak for the diaminododecane derivatized cellulose represents 39.2% of the total carbon. It must be remembered, however, that a larger portion of that carbon peak is due to surface contamination as discussed in Section 3.6.1.

3.6.3 Sodium Heparin XPS Analyses

The main elements found in N-acetylated sodium heparin were sodium, sulfur, carbon, nitrogen and oxygen. The atomic percents of sulfur, carbon, nitrogen and oxygen, neglecting sodium which was not analyzed in all other spectra, are presented in Table XV. The high resolution carbon $1S_{1/2}$ spectrum is very similar to the cellulose spectrum and is not shown. The sulfur $2S_{1/2}$ peak occurred at 232.17 eV.

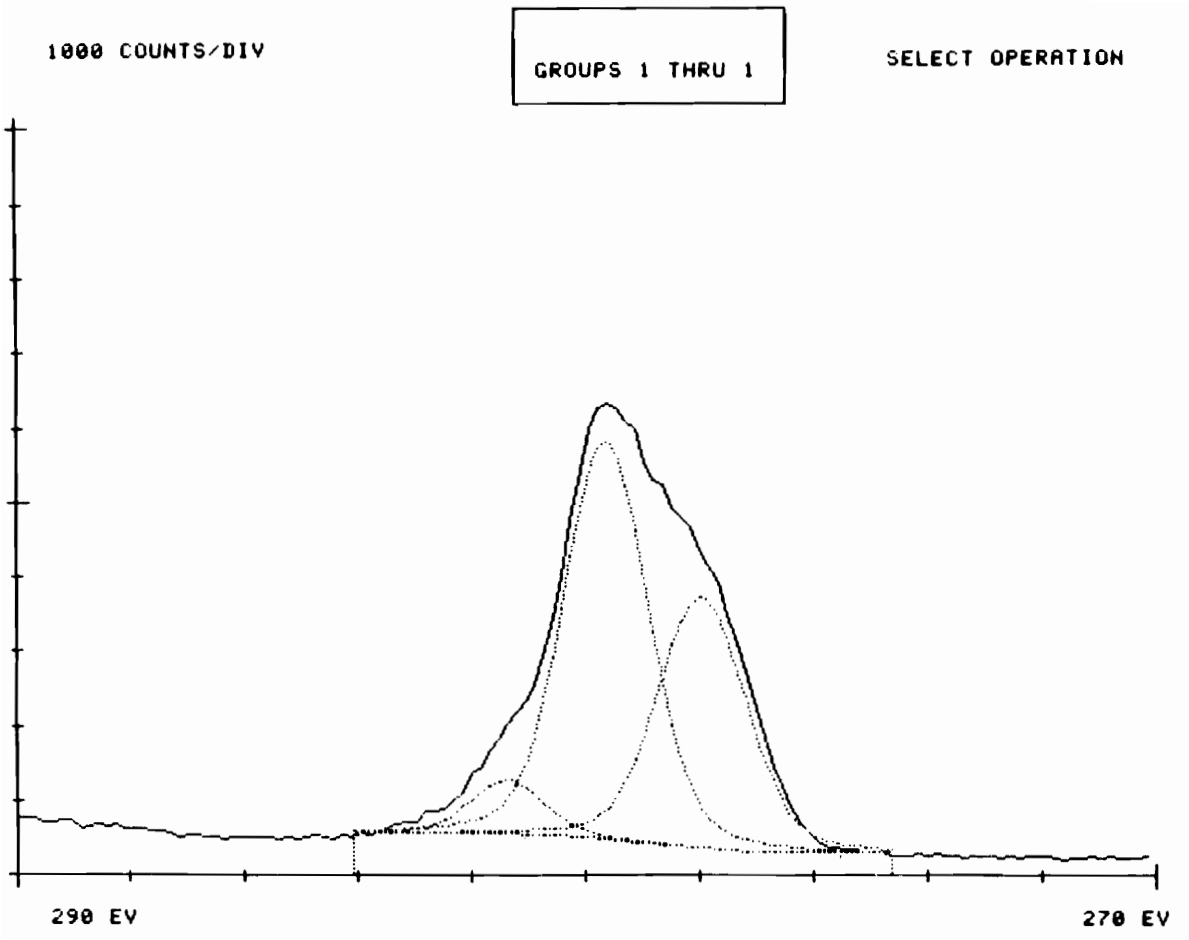


Figure 20. XPS spectrum of carbon $1S_{1/2}$ electrons for diaminobutane derivatized cellulose sheets.

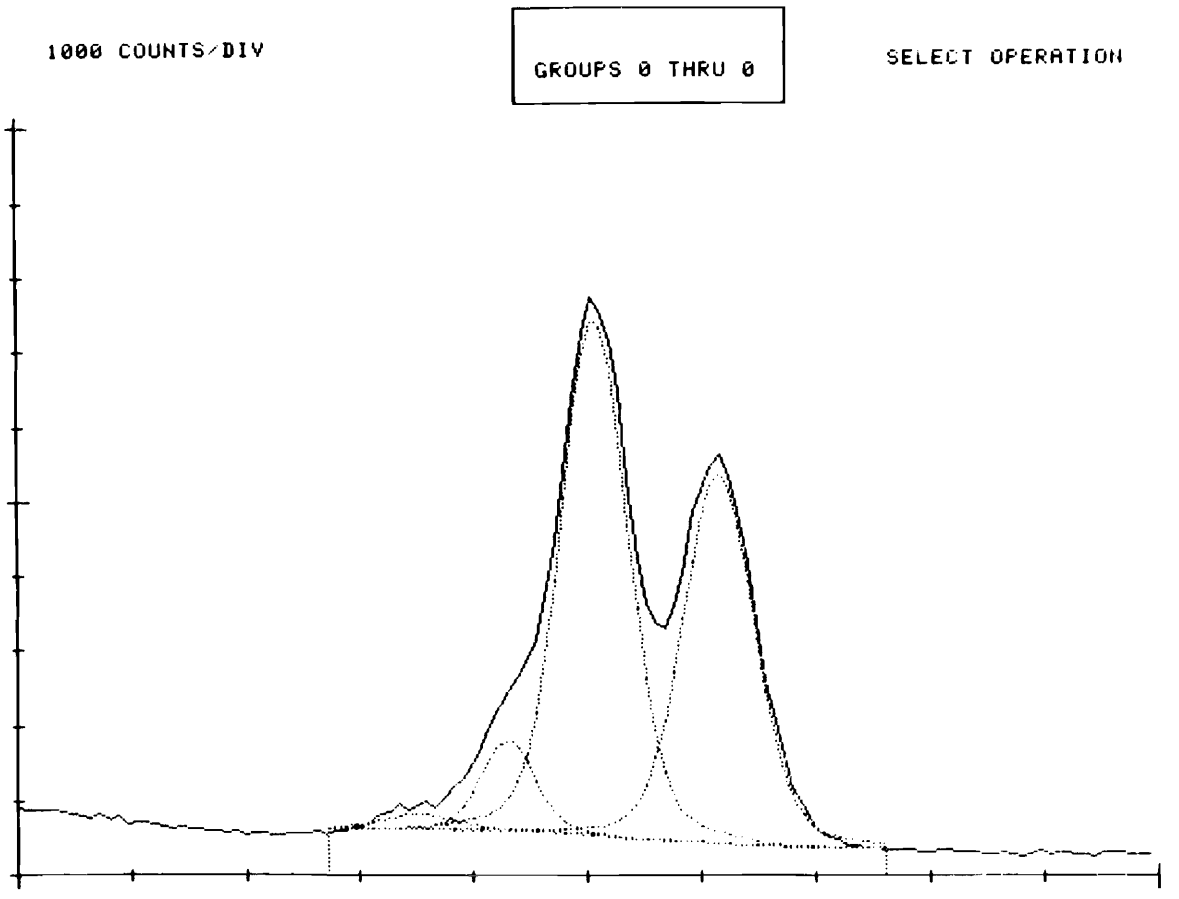


Figure 21. XPS spectrum of carbon $1S_{1/2}$ electrons for diaminododecane derivatized cellulose sheets.

Table XV

Atomic Percents for
N-acetylated Heparin

Element	Cross Section-Electron Type	Number of Scans	Atomic Percent
S	$1.43\text{-}2S_{1/2}$	10	5.3
C	$1.00\text{-}1S_{1/2}$	10	49.1
O	$2.93\text{-}1S_{1/2}$	10	43.0
N	$1.80\text{-}1S_{1/2}$	10	2.6

3.6.4 Heparin Immobilized to Diaminoalkane Derivatized Cellulose

The atomic percents of sulfur, carbon, oxygen and nitrogen for heparin immobilized to diaminoalkane derivatized celluloses are presented in Table XVI.

The high resolution carbon 1S spectra for heparin immobilized to diaminoalkane celluloses are similar to the respective spectra obtained for the diaminoalkane celluloses, both showing large alkane carbon low binding energy peaks. The high resolution sulfur $2S_{1/2}$ spectra for heparin immobilized to diaminobutane and diaminododecane celluloses are presented in Figures 22 and 23 respectively, peak maxima occurring at 231.88 and 231.57 eV respectively. The atomic percents of sulfur for the two surfaces are very low, 0.3% for both diaminobutane and diaminododecane spacer arms; however, it should be noted that pure heparin had only 5.3 atomic percent sulfur.

3.7 Activated Partial Thromboplastin Time Test

3.7.1 Functional Group Derivatized Heparins

The activities of the N-acetylated heparin and the carboxylic derivatized heparins determined by APTT tests, plus or minus 95% confidence limits, are presented in Table XVII. The activities of the hydroxyl derivatized heparins, plus or minus 95% confidence limits, are presented in Table XVIII.

Table XVI

Heparin Immobilized to Diaminoalkane Derivatized Celluloses

Spacer Arm	Element	Cross Section-Electron Type	Number of Scans	Atomic Percent
Diaminobutane	S	1.43-$2S_{1/2}$	50	0.3
Diaminobutane	C	1.00-$1S_{1/2}$	10	66.1
Diaminobutane	O	2.93-$1S_{1/2}$	10	36.6
Diaminobutane	N	1.80-$1S_{1/2}$	20	2.0
Diaminododecane	S	1.43-$2S_{1/2}$	50	0.3
Diaminododecane	C	1.00-$1S_{1/2}$	10	65.9
Diaminododecane	O	2.93-$1S_{1/2}$	10	29.7
Diaminododecane	N	1.80-$1S_{1/2}$	20	4.1

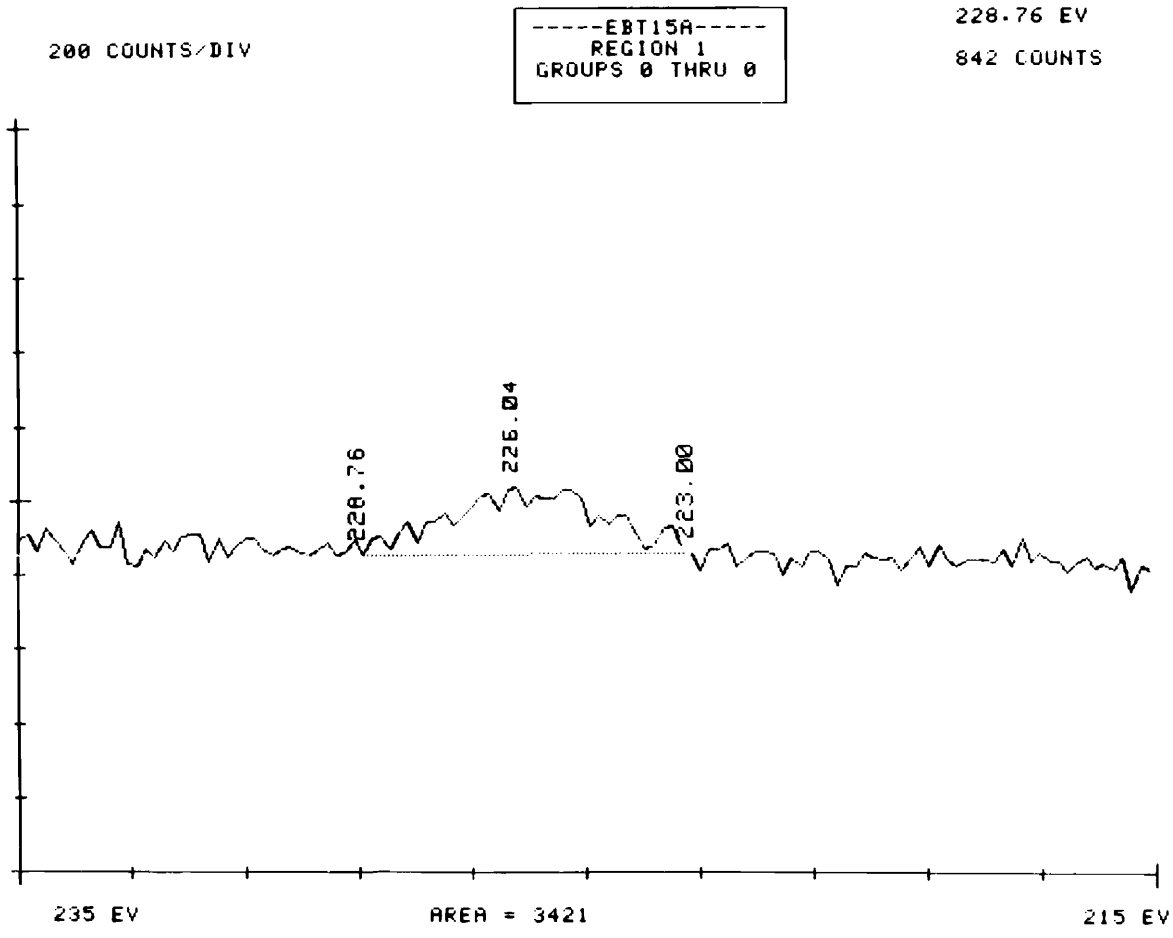


Figure 22. XPS spectrum of sulfur $2S_{1/2}$ electron for heparin coupled diaminobutane derivatized cellulose.

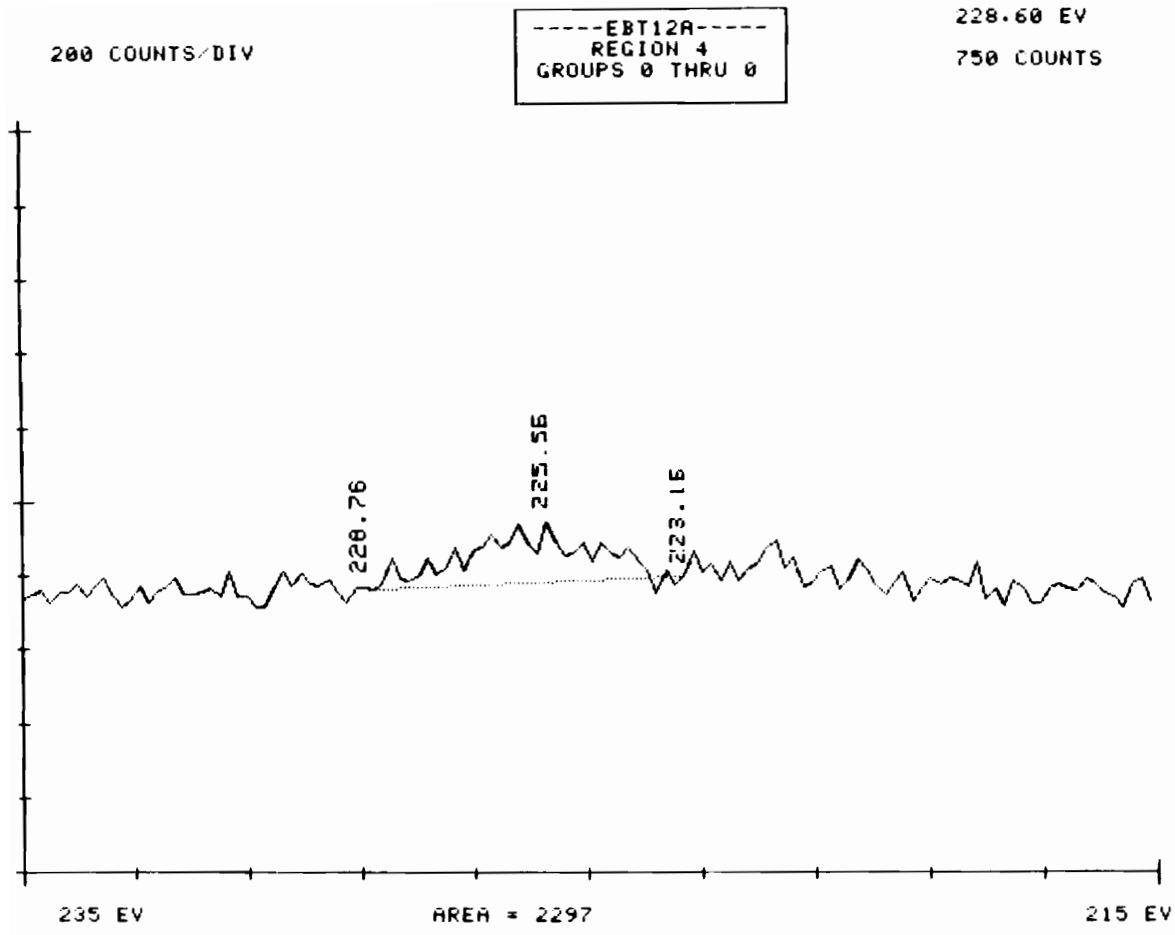


Figure 23. XPS spectrum of sulfur $2S_{1/2}$ electrons for heparin coupled diaminododecane derivatized cellulose.

Table XVII

Anticoagulant Activity of Carboxylic Group Derivatized Heparins

R (Alkyl Group)	% Derivatization	Activity (units/mg)
Control (N-acetylated)	0	157 ± 14
n-butylamine	17.9	132 ± 8
n-butylamine	36.9	128 ± 8
n-butylamine	100.0	0
2-aminoethyl hydrogensulfate	10.1	111 ± 10

Table XVIII

Activities of Hydroxyl Group
Derivatized Heparin

Crosslinking Agent	R (Alkyl Group)	%Derivatization*	Activity (units/mg)
epichlorohydrin	n-butylamine	5-30	124 ± 11
epichlorohydrin	2-aminoethyl hydrogensulfate	5-30	132 ± 5
epichlorohydrin	glycine	5-30	121 ± 8
divinylsulfone	n-butylamine	5-30	113 ± 5
divinylsulfone	2-aminoethyl hydrogensulfate	5-30	133 ± 10
divinylsulfone	glycine	5-30	130 ± 14

*Estimated based on nmr spectra.

3.7.2 Immobilized Heparin Gels

The APTT results for plasma obtained for 0.5 ml of heparin immobilized to diaminoalkane derivatized agarose gels incubated 10 minutes at room temperature with 10 ml of plasma is presented in Figure 24, the region delineated by the dashed lines represents the baseline APTT of control plasma, plus or minus one standard deviation. The open circles represent mean APTT values for heparin immobilized via different diaminoalkane spacer arm lengths, indicated by the number of carbon atoms in the respective diaminoalkane spacer arm. The solid circles represent APTT values obtained for the respective diaminoalkane-agarose without immobilized heparin, one standard deviation is represented by the bar markers on each point. Activated partial thromboplastin times for plasma obtained from 1 ml of heparin immobilized gels incubated with 10 ml of plasma follow a similar pattern; APTT for heparin immobilized via 2 and 4 carbon unit diaminoalkane spacer arms ranged between 63.5 and 81.8 seconds; however, mean APTT for the 8 and 10 carbon unit diaminoalkane spacer arms were 106.2 and 295.2 seconds respectively while heparin immobilized via the 12 carbon unit spacer arm produced APTT times greater than 30 minutes, no clotting was ever observed. When 0.5 ml of the heparin immobilized via divinylsulfone to Sepharose 4-B beads was incubated 10 minutes with plasma, the resultant supernatant plasma had an APTT of 54.1 $\pm$ 7.8 seconds, comparable to heparin immobilized to comparable length diaminoalkane gels.

Tritium levels in the above plasmas were determined by liquid

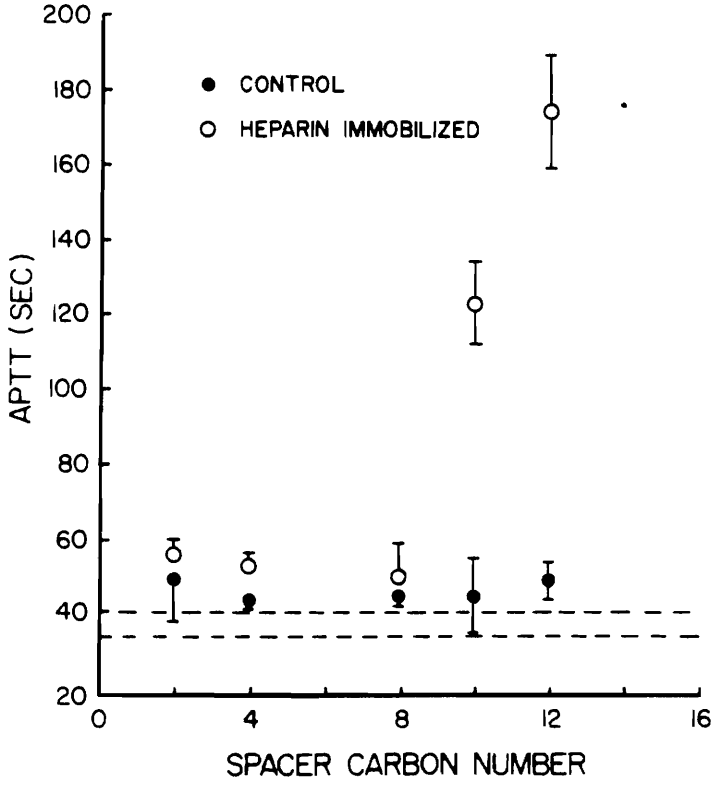


Figure 24. Activated partial thromboplastin time versus dimainoalkane carbon number.

scintillation counting. The dpm/ml, after background subtraction, and the calculated concentrations of free heparin for the above plasmas are presented in Table XIX.

Although free heparin plasma concentrations were found to increase with increased spacer arm length, the plasma dpm/ml values are only slightly over background levels. Furthermore, to produce APTT clotting times greater than 200 seconds would require a minimum of .45 units/ml of heparin. This concentration of heparin would produce 123 dpm/ml in the test plasma. Even in plasmas with APTT greater than 30 minutes, only 28.97 dpm/ml was measured. It can therefore be concluded that the increased clotting times associated with the immobilized heparin gels are not due to the release of heparin from the gels.

3.8 XPS Analyses of Heparinized Surfaces After Protein Adsorption

3.8.1 Single Component Proteins Solutions

Heparin immobilized diaminobutane and diaminododecane cellulose surfaces were exposed for 2 hours to 10 mg/ml solutions of ATIII, albumin and fibrinogen. After rinsing, the atomic percents for sulfur, carbon, nitrogen and oxygen were determined. Respective elemental atomic percents for heparin immobilized to diaminobutane and diaminododecane derivatized celluloses are presented in Tables XX and XXI.

The high resolution spectra of the sulfur $2S_{1/2}$ electrons for

Table XIX

Free Heparin Concentrations in
APTT Test Plasmas

Spacer Arm	Mean APTT (Sec)	Plasma Heparin Concentration (dpm/ml)	Plasma Heparin* Concentration (units/ml)
Diaminoethane	57.0	7.47	.03
Diaminobutane	53.4	12.18	.04
Diaminooctane	51.4	11.42	.04
Diaminodecane	124.1	8.41	.03
Diaminodecane	295.2	15.05	.05
Diaminododecane	174.9	22.52	.08
Diaminododecane	>1500	28.97	.11
Divinylsulfone	54.1	13.03	.05

*Based on a specific activity of 43,114 dpm/mg and an anticoagulant activity of 157 units/mg.

Table XX

Atomic Percents for Heparinized
Diaminobutane Cellulose Surface

Protein Solution	Element	Cross Section-Electron Type	Number of Scans	Atomic Percent
ATIII	S	$1.43\text{-}2S_{1/2}$	50	0
	C	$1.00\text{-}1S_{1/2}$	10	63.7
	N	$1.80\text{-}1S_{1/2}$	10	6.3
	O	$2.93\text{-}1S_{1/2}$	10	30.0
Albumin	S	$1.43\text{-}2S_{1/2}$	50	0.2
	C	$1.00\text{-}1S_{1/2}$	10	65.3
	N	$1.80\text{-}1S_{1/2}$	10	4.7
	O	$2.93\text{-}1S_{1/2}$	10	29.8
Fibrinogen	S	$1.43\text{-}2S_{1/2}$	50	0.2
	C	$1.00\text{-}1S_{1/2}$	10	66.5
	N	$1.80\text{-}1S_{1/2}$	20	4.2
	O	$2.93\text{-}1S_{1/2}$	10	29.1

Table XXI

Atomic Percents for Heparinized
Diaminododecane Cellulose Surfaces

Protein Solution	Element	Cross Section-Electron Type	Number of Scans	Atomic Percent
ATIII	S	$1.43\text{-}2S_{1/2}$	50	0
	C	$1.00\text{-}1S_{1/2}$	10	63.8
	N	$1.80\text{-}1S_{1/2}$	10	5.2
	O	$2.93\text{-}1S_{1/2}$	10	31.0
Albumin	S	$1.43\text{-}2S_{1/2}$	50	0.2
	C	$1.00\text{-}1S_{1/2}$	10	64.0
	N	$1.80\text{-}1S_{1/2}$	10	6.3
	O	$2.93\text{-}1S_{1/2}$	10	29.5
Fibrinogen	S	$1.43\text{-}2S_{1/2}$	50	0.1
	C	$1.00\text{-}1S_{1/2}$	10	65.1
	N	$1.80\text{-}1S_{1/2}$	20	3.7
	O	$2.93\text{-}1S_{1/2}$	10	31.1

heparin immobilized to diaminobutane surfaces exposed to 10 mg/ml solutions of ATIII, albumin and fibrinogen are shown in Figures 25, 26 and 27 respectively. It is readily seen that no sulfur $2S_{1/2}$ peaks occur near 232 eV as was the case for the heparin powder sulfur $2S_{1/2}$ electrons and for the immobilized heparin surfaces prior to protein adsorption (Figures 22 and 23). Small peaks do occur at $\sim$ 226.5 eV for the protein adsorbed heparinized diaminobutane surface. The sulfur in heparin is as either a sulfate or sulfamate group, therefore heparin sulfur appears at a higher binding energy due to the high electronegative environment of the sulfur atoms in the sulfate and sulfamate groups. The sulfur peaks observed at $\sim$ 226.5 eV in Figures 25, 26 and 27 cannot be from heparin sulfur and must therefore arise from sulfur in cysteine and cystine residues in the adsorbed proteins. The fact that no sulfur peaks are observed at $\sim$ 232 eV can only be interpreted as evidence that heparin was not associated with the surface of the protein adsorbates. Rather, the immobilized heparin is covered by the absorbed protein layer. The high resolution sulfur $2S_{1/2}$ spectra for the heparinized diaminododecane derivatized surfaces after contact with ATIII, albumin and fibrinogen solutions are present in Figures 28, 29 and 30 respectively. No peaks are observed near 232 eV, small peaks are observed at $\sim$ 226.5 eV. The same argument applies with these spectra. Heparin sulfur is not present on the surface of the adsorbed protein layers, what sulfur does appear must originate from the adsorbed proteins. This can only mean that the immobilized

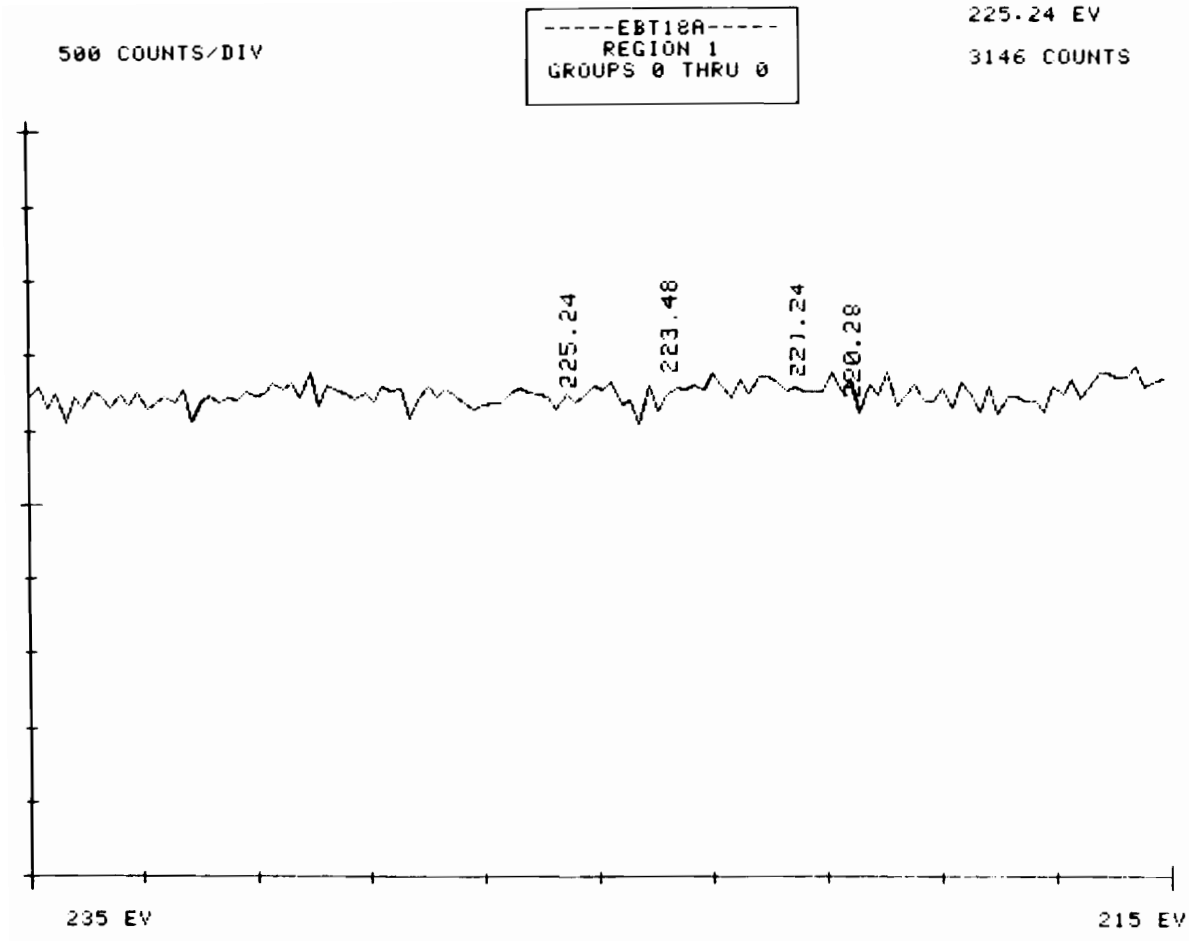


Figure 25. XPS spectrum of sulfur $2S_{1/2}$ electrons for heparin coupled to diaminobutane derivatized cellulose after antithrombin III adsorption.

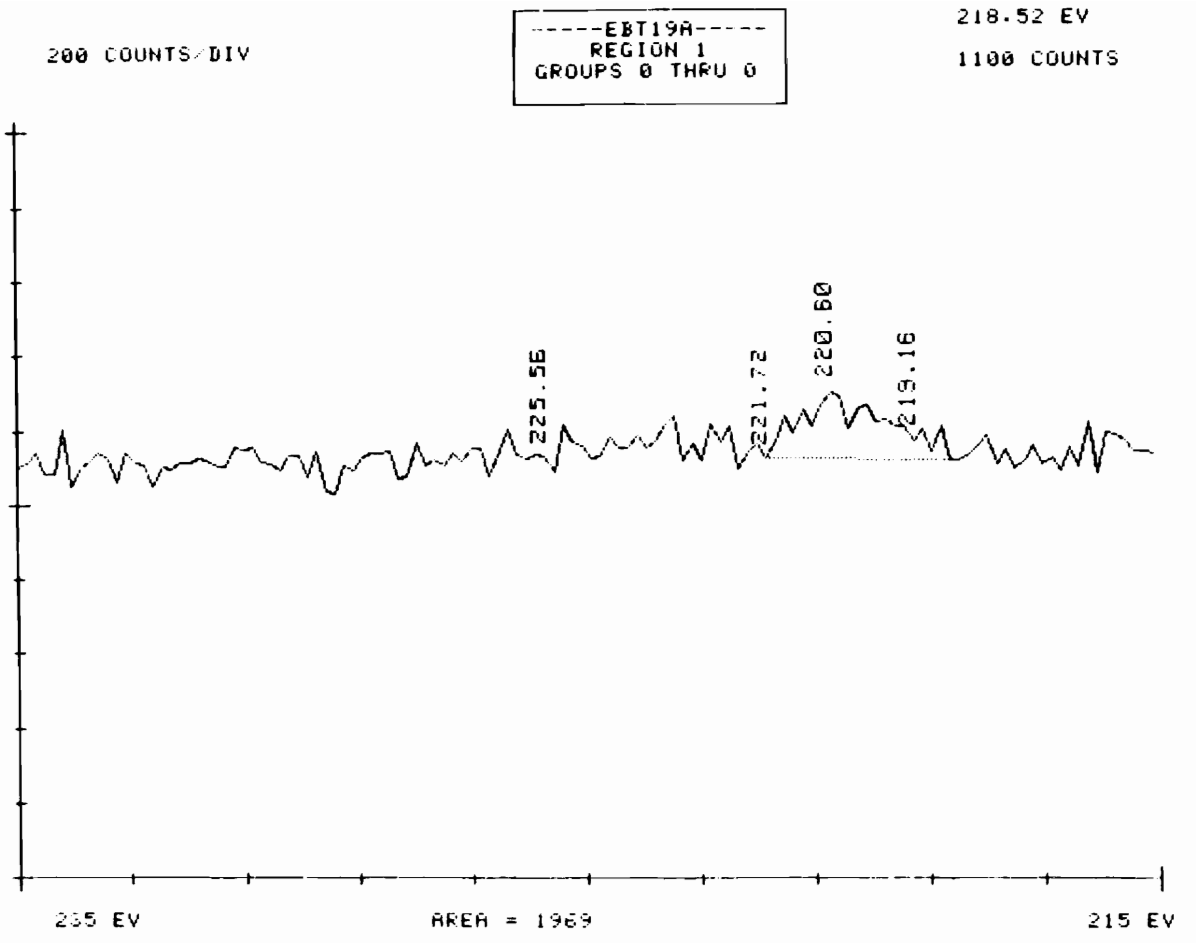


Figure 26. XPS spectrum of sulfur $2S_{1/2}$ electrons for heparin coupled to diaminobutane derivatized cellulose after albumin adsorption.

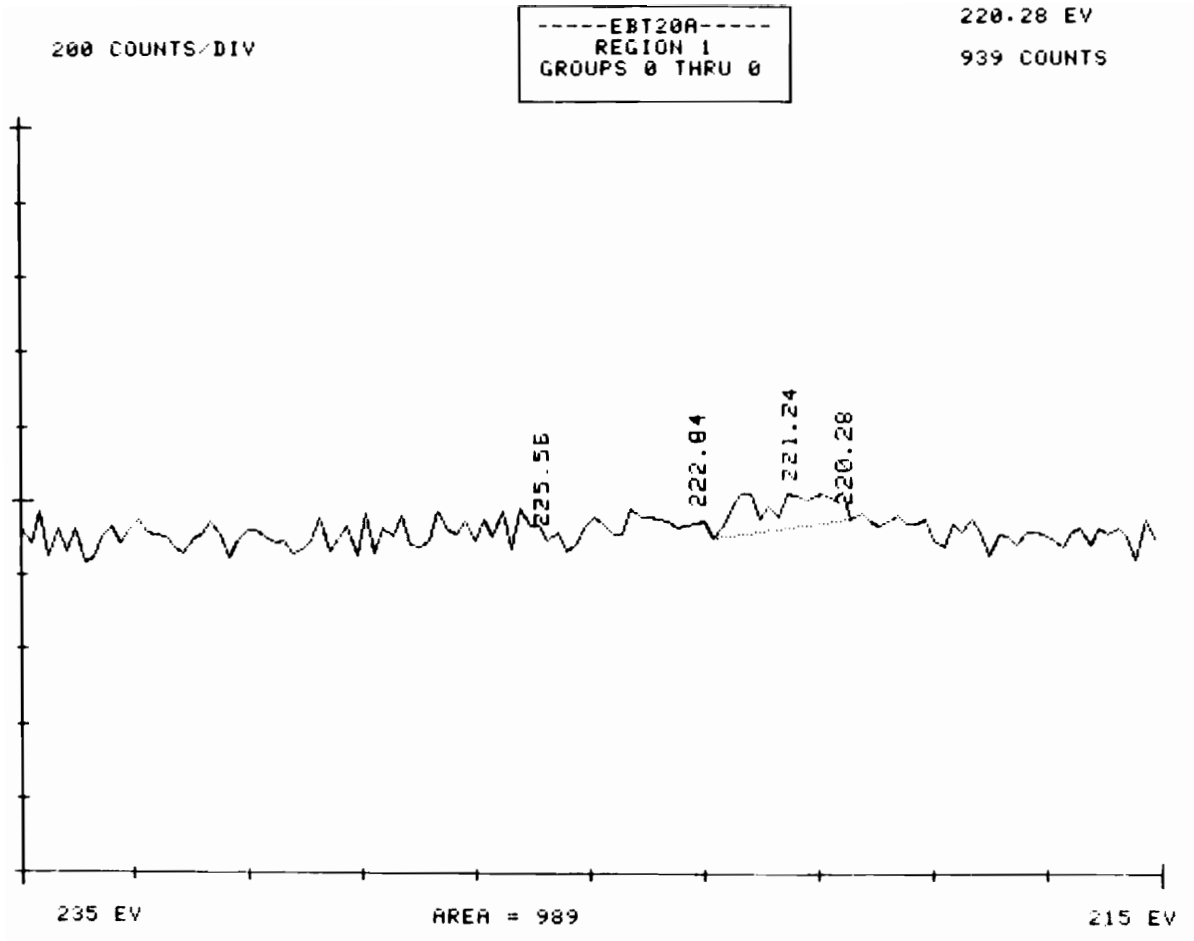


Figure 27. XPS spectrum of sulfur $2S_{1/2}$ electrons for heparin coupled to diaminobutane derivatized cellulose after fibrinogen adsorption.

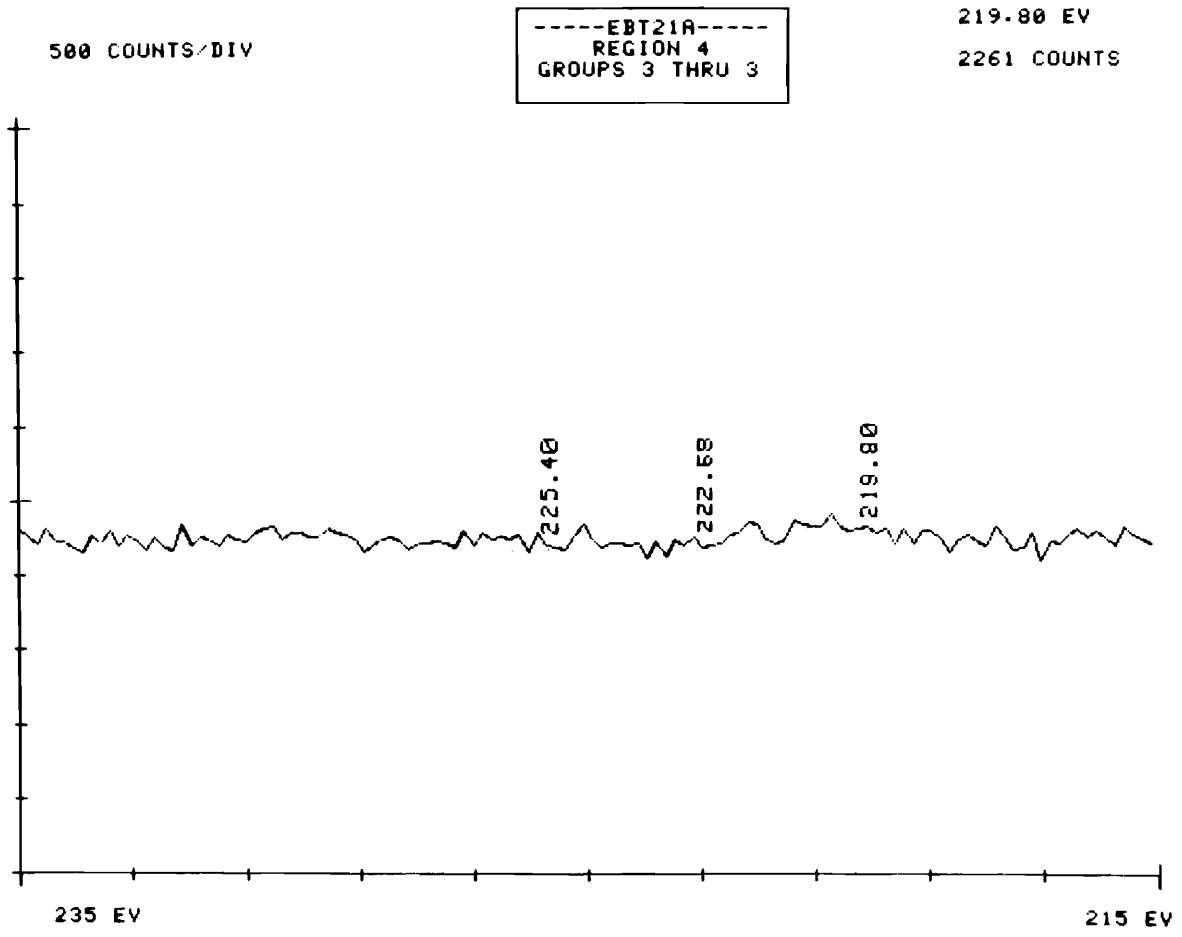


Figure 28. XPS spectrum of sulfur $2S_{1/2}$ electrons for heparin coupled to diaminododecane derivatized cellulose after antithrombin III adsorption.

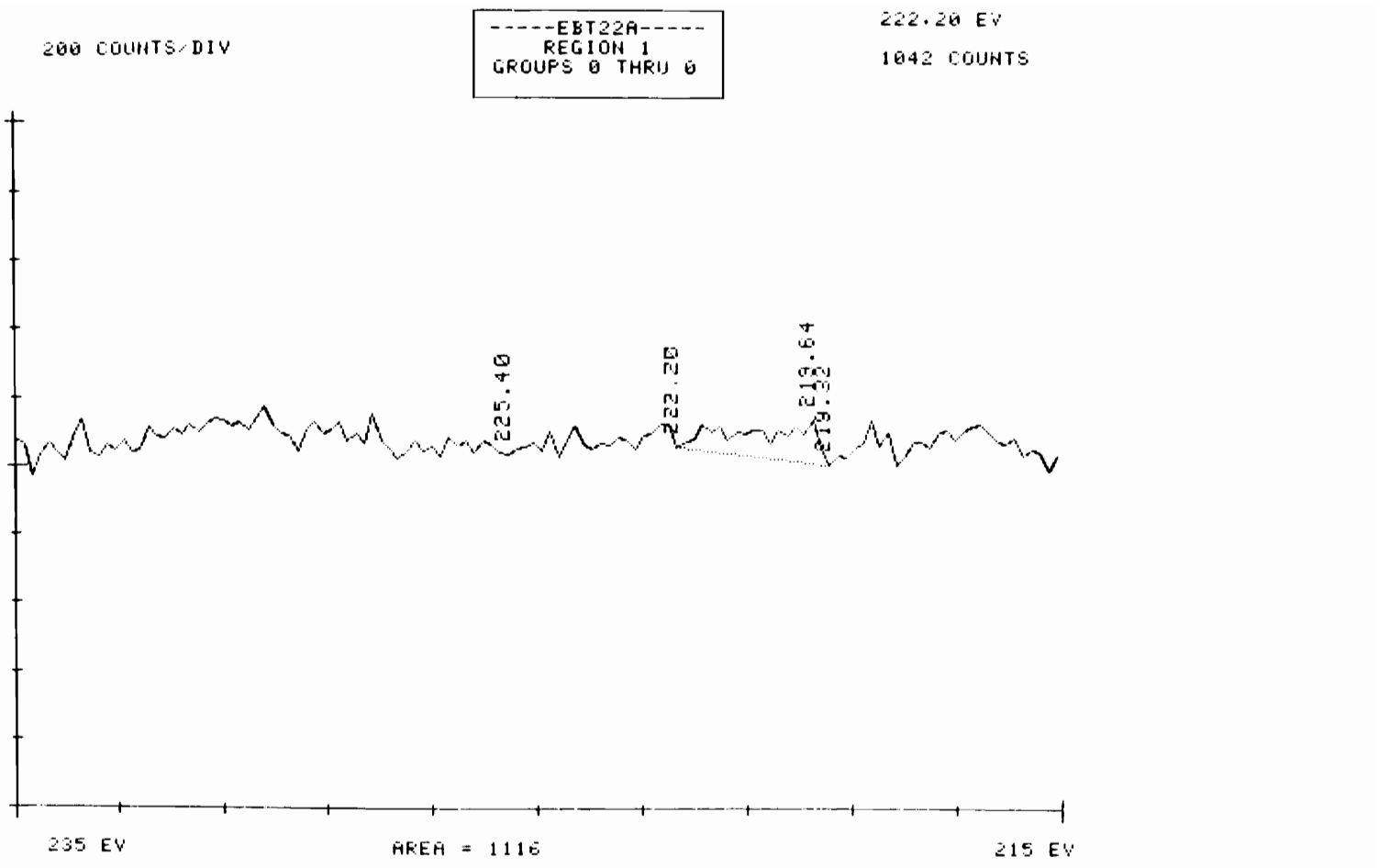


Figure 29. XPS spectrum of sulfur $2S_{1/2}$ electrons for heparin coupled to diaminododecane derivatized cellulose after albumin adsorption.

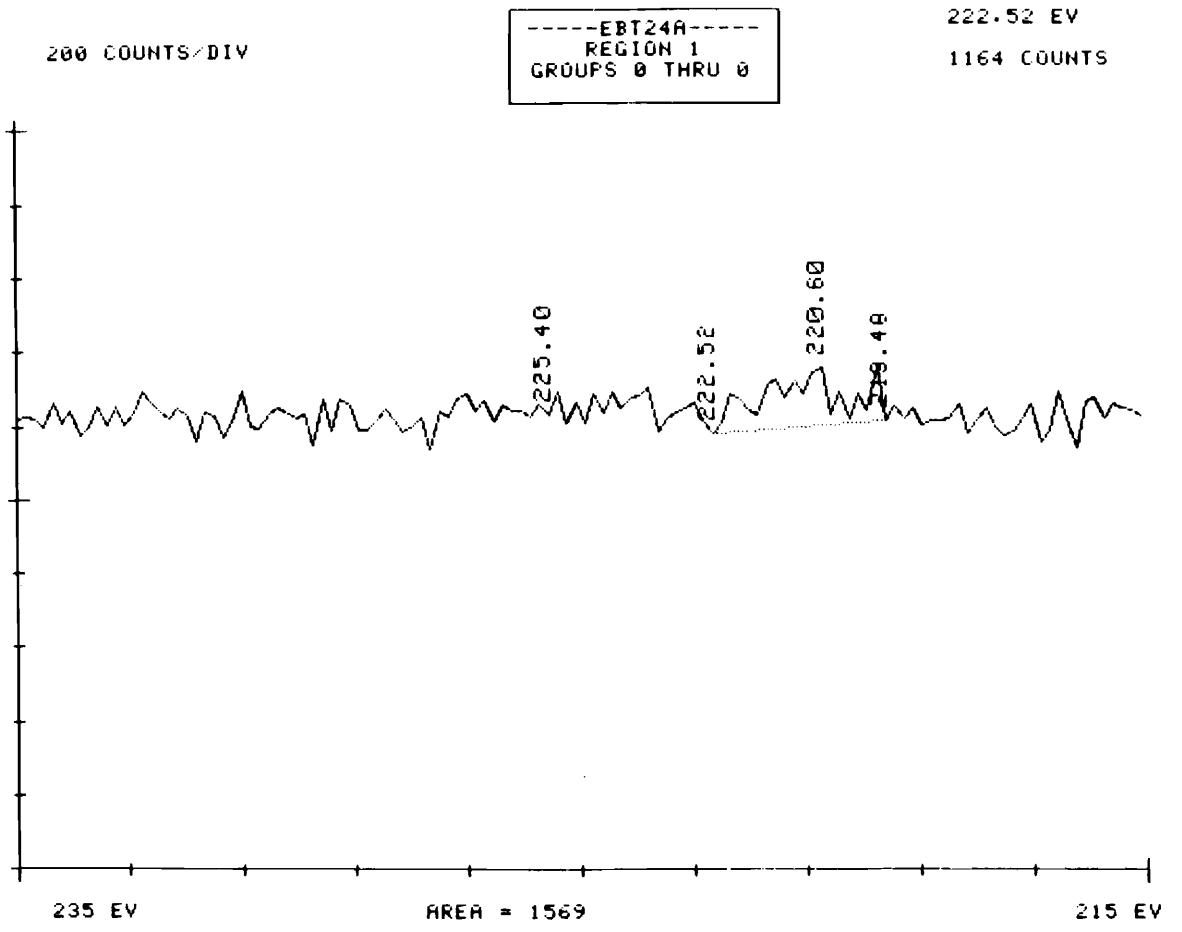


Figure 30. XPS spectrum of sulfur $2S_{1/2}$ electrons for heparin coupled to diaminododecane derivatized cellulose after fibrinogen adsorption.

heparin is covered by the adsorbed protein.

3.8.2 Heparinized Surfaces Exposed to Plasma

The elemental atomic percents calculated for heparin immobilized to diaminobutane and diaminododecane derivatized cellulose surfaces after 2 hours exposure to plasma and subsequent rinsing are presented in Table XXII.

The heparin immobilized to the diaminobutane cellulose, after plasma protein adsorption, produced the largest nitrogen content of any surface evaluated; almost twice the atomic percent of nitrogen. As with the prior section on protein adsorption from single component solutions, no sulfur $2S_{1/2}$ peaks are observed near 232 eV; instead, small peaks at $\sim$ 277 eV are observed. Once again, this can only be interpreted as evidence that heparin was not associated with the surface of the protein adsorbate layers and must therefore be masked underneath the adsorbed proteins. The high resolution sulfur $2S_{1/2}$ spectra for heparinized diaminobutane and diaminododecane derivatized celluloses after plasma contact are presented in Figures 31 and 32 respectively.

3.9 Platelet Retention and PF4 Release

The fraction of platelets retained in the 1 cc gel volume of heparin immobilized diaminobutane and diaminododecane Sepharose 6-MB gels are presented in Figure 33, the marker bars indicate one standard deviation. Platelet retention for diaminobutane and diaminododecane Sepharose 6-MB gels without immobilized heparin

Table XXII

Atomic Percents of Heparinized Surfaces After Plasma Contact

Diaminoalkane Spacer Arm	Element	Cross Section-Electron Type	Number of Scans	Atomic Percent
Diaminobutane	S	1.43-2S	50	0.2
	C	1.00-1S	10	66.1
	N	1.80-1S	10	11.5
	O	2.93-1S	10	22.2
Diaminododecane	S	1.43-2S	50	0.1
	C	1.00-1S	10	69.7
	N	1.80-1S	10	7.7
	O	2.93-1S	10	22.5

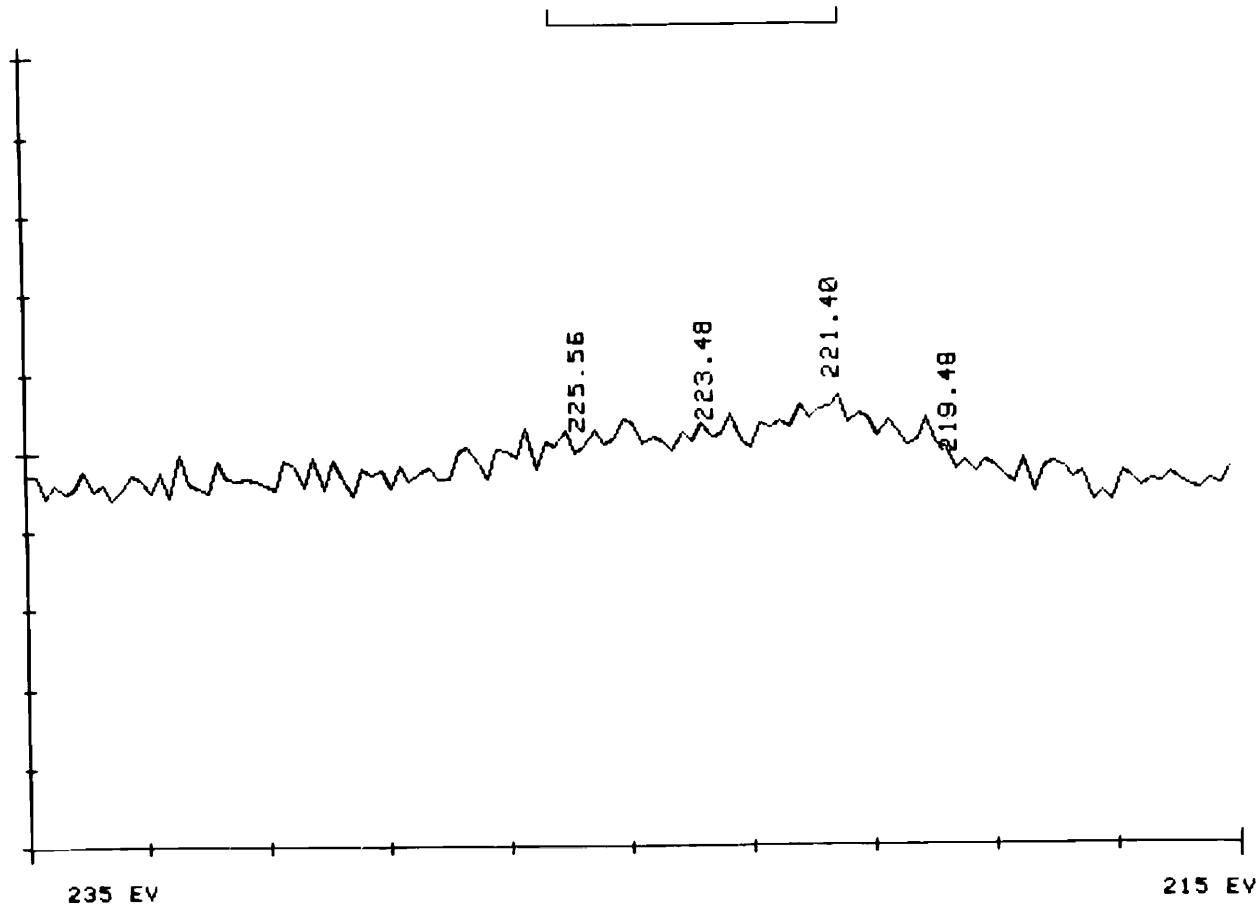


Figure 31. XPS spectrum of sulfur $2S_{1/2}$ electrons for heparin coupled to diaminobutane derivatized cellulose after plasma contact.

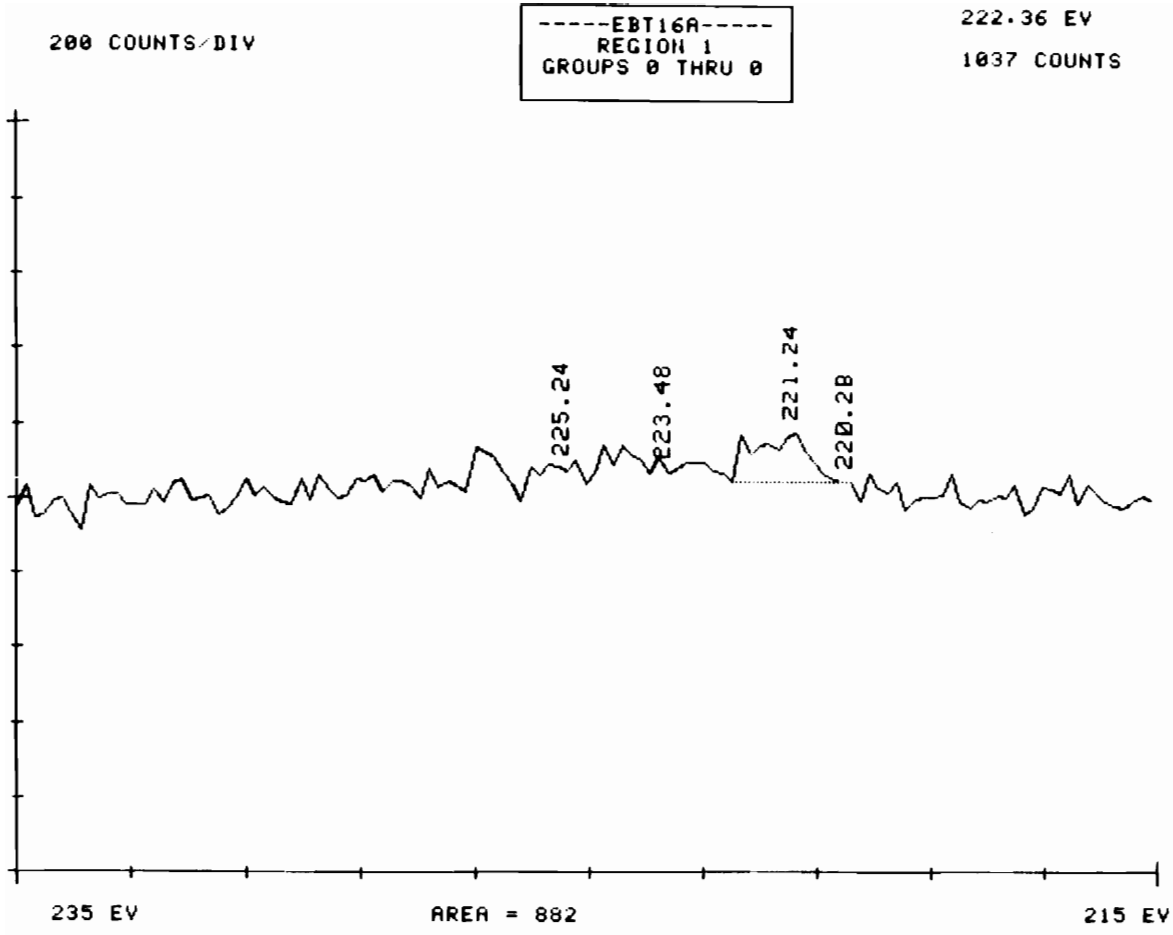


Figure 32. XPS spectrum of sulfur $2S_{1/2}$ electrons for heparin coupled to diaminododecane derivatized cellulose after plasma contact.

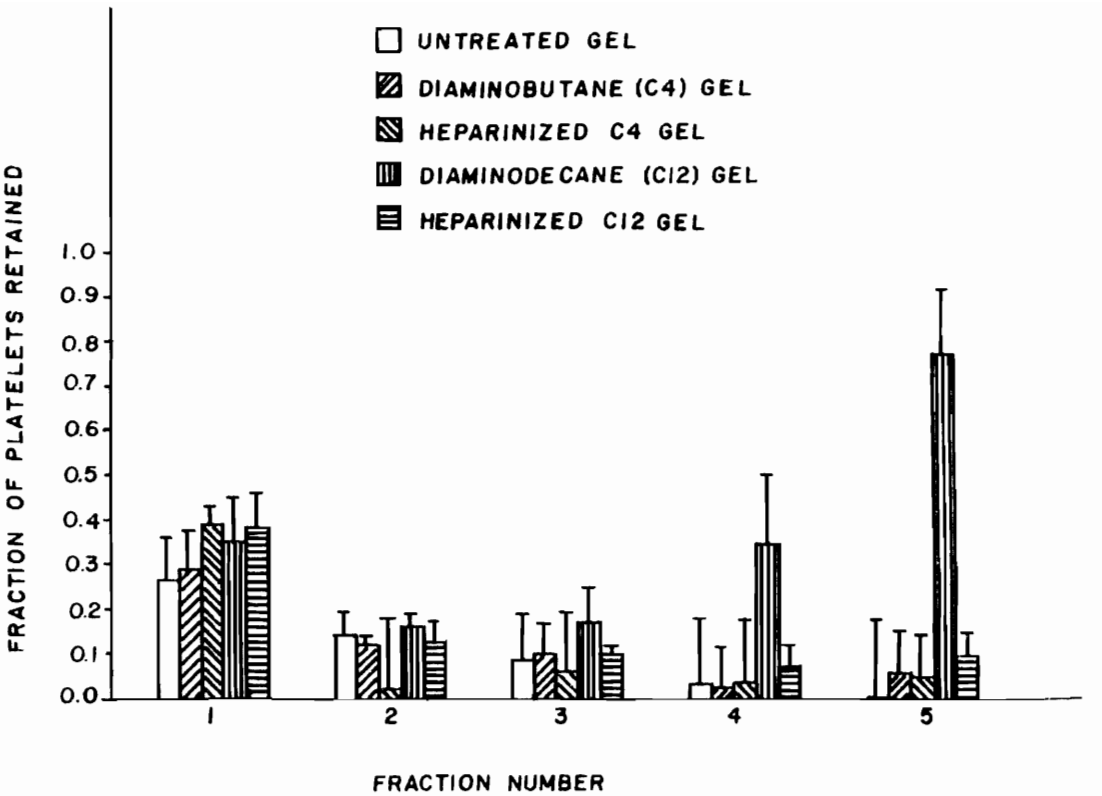


Figure 33. Fraction of platelets retained versus fraction number for various gels.

and untreated Sepharose 6-MB gels are also presented. The fraction of platelets retained was calculated from the equation:

$$\text{Fraction Retained} = 1 - \left[\frac{(\#\ \text{platelets}/_{mm}3)\ \text{test sample}}{(\#\ \text{platelets}/_{mm}3)\ \text{baseline control}} \right]$$

The initially high values for platelet retention associated with all materials in fraction #1 corresponds with hematocrit dilution; however, beginning with fraction #4, platelet retention for the diaminododecane derivatized gel significantly increases until 76.8% $\pm$ 14.4% of the platelets are retained in the final whole blood fraction. These column were clotted at the end of the 25 ml whole blood perfusion experiments. No significant differences in platelet retention were observed for any of the other gels. Platelet factor 4 levels in plasmas from whole blood fractions and in rinse buffers for the above gels are presented in Figure 34. For a given fraction, untreated Sepharose 6-MB produced the least amount of PF4 release.

The differences in PF4 release for the various fractions are statistically insignificant between the diaminobutane derivatized Sepharose 6-MB and both heparin immobilized Sepharoses, and these levels are only slightly higher than the corresponding fractions obtained with untreated Sepharose. However, all fractions collected from the diaminododecane derivatized gels show significantly higher levels of PF4 than corresponding fractions for all other gels. Platelet factor 4 levels are approximately three times higher in all whole blood fractions obtained for the diaminododecane derivatized Sepharose than corresponding fractions

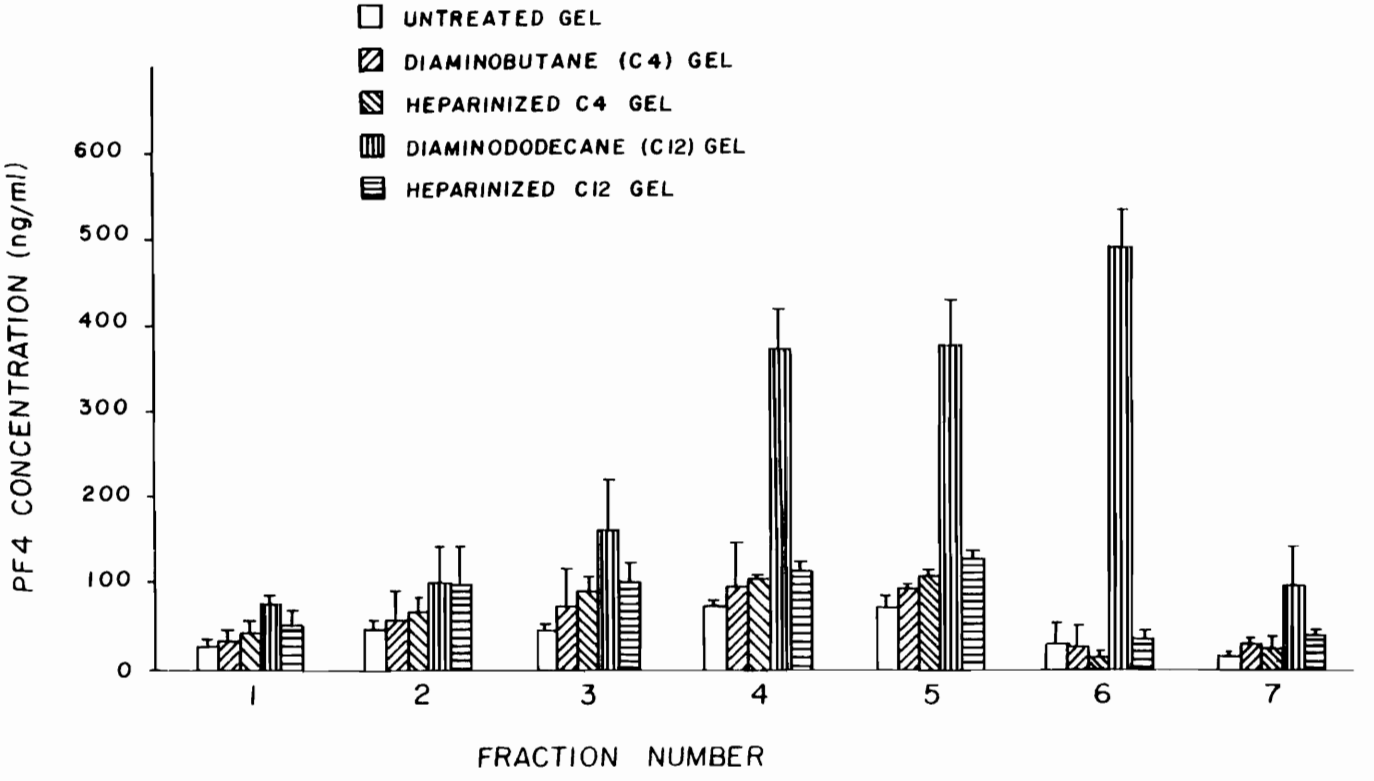


Figure 34. Amount of platelet factor 4 released in collected fractions for various gels.

from other gels, and these elevated PF4 levels correspond with the increased platelet retention beginning with fraction #4.

CHAPTER 4

DISCUSSION

4.1 Functional Group Derivatization

Free amine groups of heparin can be blocked with no loss in anticoagulant activity. With this prior knowledge, all carboxylic and hydroxyl group derivatizations were subsequently conducted with N-acetylated heparin to prevent intra- and intermolecular crosslinking. It was found that the heparin carboxylic groups could be partially derivatized with only minimal losses in anticoagulant activity; however, the degree of derivatization was critical at a certain level. More than 50% derivatization with n-butylamine resulted in the complete loss of anticoagulant activity. Heparin carboxylic groups have been blocked by others (98), and those derivatized heparins possessed no anticoagulant activity. However, in those studies, only fully derivatized compounds were investigated and partially derivatized compounds were not evaluated. Interestingly, the end group on the derivatization ligand did not appear to greatly influence the resultant anticoagulant activity. Both methyl end groups, n-butylamine reactions, and sulfate end groups, 2-aminoethyl hydrogen sulfate reactions, on respective carboxylic derivatized heparins showed anticoagulant activity under partial derivatization.

The same trend was observed for the hydroxyl derivatization reactions using two different crosslinking agents, epichlorohydrin and divinylsulfone. Although the degree of derivatization was not further investigated, partially derivatized heparins were found to be active; and the derivatization ligand end groups once again did not appear to greatly influence the resultant anticoagulant activity. Methyl end groups, n-butylamine reactions, sulfate end groups, 2-aminoethyl hydrogen sulfate reactions, and carboxylic end groups, glycine reactions, on hydroxyl derivatized compounds all produced active heparin under partial derivatization conditions. Since these hydroxyl derivatization reactions were conducted with crosslinking agents, heparin crosslinking was a possibility that could not be dismissed.

Because crosslinking would be a difficult parameter to control, the majority of immobilization reactions were conducted utilizing heparin carboxylic groups; although heparin immobilization was endeavored via heparin hydroxyl groups using the crosslinking agent divinylsulfone. Results from the n-butylamine derivatization of heparin carboxylic groups demonstrated that active immobilized heparin could be achieved were heparin immobilized to aminoalkane spacer arms and if the number of heparin carboxylic groups participating in the amidation reaction was minimized. Amidation reactions with n-butylamine demonstrated that derivatized heparin was still active if approximately 20% of the total carboxylic groups were blocked. All heparin immobilization reactions, involving the formation of an amide linkage be-

ween heparin carboxylic groups and free surface associated amine groups, were conducted using sufficient quantitites of Woodward's Reagent K to activate a maximum of 20% of the total carboxylic groups assuming 100% efficiency in the overall immobilization scheme.

4.2 Heparin Immobilization

4.2.1 Heparin Immobilization to Diaminoalkane Derivatized Agarose Beads

Using reaction conditions such that a maximum of 20% of the heparin carboxylic groups participated in the immobilization reactions, heparin was coupled to agarose beads via different length diaminoalkane derivatized agarose beads. The degree of heparin coupling to the diaminoalkane-agaroses appeared dependent upon the hydrocarbon length of the diaminoalkane spacer arm. Low degrees of heparin coupling were observed for a 2 carbon diaminoalkane spacer arm (see Tables IV and VII). Heparin coupling, relative to the surface concentration of diaminoalkane spacer arms, progressively increased with increased spacer arm length up to the 8 carbon spacer arm, afterwhich immobilization progressively decreased with the 10 and 12 carbon spacer arms. The anticoagulant activity of the immobilized heparin, as determined by APTT methods, also appears dependent upon the spacer arm length. As shown in Figure 24 and in Table XIX, APTT was slightly greater than baseline control plasma APTT for heparin immobilized via 2, 4 and 8 carbon spacer arms; however, beginning with the 10 carbon spacer

arm, APTT significantly increases over baseline values with increasing spacer arm length. The increased clotting time response was dose dependent and higher clotting times could be achieved with greater surface areas. This increased APTT for plasmas exposed to immobilized heparin surfaces could not be attributed to the release of free heparin. Scintillation counting of tritium labeled heparin in plasmas exposed to the immobilized heparin materials revealed some free heparin; however, those levels were far too low to produce the observed levels of prolonged clotting. Slightly elevated APTT times were also observed for plasmas exposed to heparin immobilized to Sepharose 4B beads via divinylsulfone, and these clotting times were similar to those observed for heparin immobilized to diaminoalkane-agaroses of comparable spacer arm length (see Table XIX). A question that arises is: are the increased clotting times due to "solution-like" heparin interactions, interactions with ATIII and activated coagulation factors, or are the increased APTT due to nonspecific adsorption of clotting factors onto a highly negatively charged surface? Larsson, et al. (99) have shown clotting times to increase for plasmas exposed to sulfated surfaces; however, clotting times for plasmas with free sulfate, added in various concentrations, was not affected over a 1.5×10^{-2} M to 1.5×10^{-9} M concentration range. Furthermore, the elevated clotting times associated with plasma exposed to the sulfate surface could be neutralized by protamine, even though no heparin was used in the experiments.

One might conclude that the increased clotting times might be

the result of this nonspecific coagulation factor depletion of the plasma following contact with the immobilized heparin materials. However, the dramatically increased clotting times associated with longer spacer arms must arise through more specific heparin interactions. Schmer (84) provided evidence that immobilized heparin had better ATIII binding and subsequent thrombin neutralization for heparin immobilized via putrescine and ε-amino capryol spacer arms. Perhaps this is what has occurred with the heparin immobilized via 10 and 12 carbon diaminoalkane spacer arms. The conclusion should not be drawn, however, that further increasing the length of the diaminoalkane spacer arm will necessarily result in still greater anticoagulant increases for immobilized heparin materials. With longer hydrocarbon diaminoalkane spacer arms, the surface becomes progressively more hydrophobic and wetting of the surface by the aqueous phase plasma will decrease. Also with longer hydrocarbon chains, folding can result and the actual spacer arm length may be substantially shorter than expected. These considerations have been addressed by Kim, et al. (100) who immobilized urokinase to Sepharose beads via different length aminoalkyl acid spacer arms. They too observed low immobilization yields with shorter spacer arms, and the immobilization yield progressively increased with longer hydrocarbon lengths. The activity of the immobilized enzymes was also found to increase with longer aminoalkyl acid spacer arms; however, a maximum activity was observed with 8 carbon spacer arms and activity progressively decreased with longer spacer arms. Those authors attri-

buted this phenomena to the increased surface hydrophobicity with the 10 and 12 carbon spacer arms; but with the highly negatively charged heparin, the surface hydrophobicity associated with 10 and 12 carbon diaminoalkane spacer arms may be negated.

The significant difference between the immobilization of heparin reported in this dissertation and prior heparin immobilization work reported in the literature lies in the control of the immobilization chemistry and in the characterization of spacer arm affects on the anticoagulant activity of the immobilized heparin. In this study, thorough characterization of the immobilization affects on the anticoagulant activity of heparin was evaluated with the soluble heparin derivatization experiments. No other group has investigated the possible alterations on the anticoagulant activity that could result from immobilizing heparin via specific functional groups on heparin and tailored the immobilization chemistry to simulate those control experiments where the anticoagulant activity was preserved. Also, prior investigations on immobilized heparin have not gone to such extremes to prevent heparin crosslinking during the immobilization reaction. By previously blocking primary amine groups on the heparin, crosslinking during the amidation coupling reactions is essentially eliminated. Numerous other prior investigations (78,79,88) have actually relied on heparin crosslinking to immobilize the heparin to the respective surfaces, yet no attempts were made to characterize the degree of crosslinking or the affect of such crosslinking on the anticoagulant activity of heparin. In addition, previous studies

in heparin immobilization have not systematically evaluated spacer arm effects on immobilized heparin activity, with the exception of Schmer, et al. (84). However, in those studies different types of spacer arms rather than different lengths of common spacer arms were studied.

All further testing of immobilized heparin surfaces was conducted on heparin immobilized to diaminobutane and diaminododecane derivatized surfaces, these being representative short and long spacer arms producing slightly elevated and greatly elevated clotting times respectively.

Although *in vitro* clotting times can be greatly increased by the presence of immobilized heparin, the thromboresistance of such surfaces *in vivo* remains in question. Platelet interactions with the immobilized heparin materials can control the overall thromboresistance. As stated in the Introduction, soluble heparin adversely affects platelets, resulting in aggregation and platelet release reactions. Plasma protein/surface interactions and platelet interactions with heparin immobilized to 4 carbon and 12 carbon diaminoalkane derivatized polymer was therefore investigated. Protein interactions with the immobilized heparin was investigated by XPS analysis of heparin surfaces, before and after exposure to protein solutions. Platelet retention and concomitant PF4 release were also investigated as described in Section 2.12. All XPS analyses were conducted on flat geometry materials while all platelet studies were conducted on Sepharose 6-MB beads.

4.2.2 XPS Analysis

XPS analysis of the starting polymer substrate, cellulose, the diaminoalkane derivatized celluloses, heparin immobilized diaminoalkane celluloses and pure heparin powder was initially conducted. The low binding energy portions of the carbon 1S electrons progressively increased with diaminobutane and diaminododecane derivatization, as expected, and the sulfur 2S electrons were used as the "heparin tag." By that, heparin sulfur is present as either a sulfate or sulfamate. The sulfur 2S electrons on heparin therefore have higher binding energies due to the high electronegative environment for the heparin sulfur atoms. Sulfur 2S electrons for heparin powder and for immobilized heparin surfaces occur at approximately 232 eV binding energy. No other sulfur atoms from proteins adsorbed onto heparinized surfaces should have 2S electron near 232 eV, rather they will occur at lower binding energies near 227 eV.

Both heparinized cellulose sheet materials demonstrated $\sim$ 0.25 atomic percent sulfur. When compared to a pure heparin powder surface, 5.29 atomic percent sulfur, it appeared that there is approximately 5-10% heparin surface coverage with both immobilized heparin materials. This low value is reflected also by tritium labeled heparin immobilization experiments where immobilization yields for heparin immobilization to flat geometry diaminoalkane-cellulose materials was also low relative to the bead geometries (see Tables VII, VIII and IX).

After two hours contact with single component ATIII, albumin

and fibrinogen solutions, no sulfur 2S electron peaks are observed near 232 eV. No sulfur 2S electron peaks are observed for heparinized diaminobutane or diaminododecane cellulose after ATIII adsorption. A very small sulfur 2S electron peak is observed for both heparinized materials near 227 eV after fibrinogen adsorption ($\sim$ 0.1 atomic percent sulfur) and a slightly larger sulfur 2S electron peak is also observed near 227 eV after albumin adsorption onto both heparinized materials ($\sim$ 0.2 atomic percent sulfur). No heparin sulfur was ever observed following protein adsorption from single component solutions. It must be concluded that heparin was not associated with the surfaces of the protein adsorbates and is instead covered by the adsorbed protein layer. It must be noted, however, that these results were obtained for dehydrate surfaces and hydrate surfaces might produce different results. The same phenomena was observed for heparinized diaminobutane and diaminododecane celluloses after plasma contact. No heparin sulfur 2S electron peaks are observed at 232 eV as was the case prior to plasma contact. Small sulfur 2S peaks are observed near 227 eV for both heparin materials with 0.2 and 0.1 atomic percents sulfur for the 4 carbon and 12 carbon spacer arms, respectively. Once again, heparin sulfur was not detected on the surface of the protein adsorbates, and it may be concluded that the immobilized heparin was covered by the adsorbed proteins. These findings are contradictory to the Auger Electron Spectroscopy analyses of Eriksson, et al. (82). After plasma protein adsorption onto their immobilized heparin surfaces,

those investigators observed sulfur on the surface of the protein adsorbate, which they attributed to heparin migrating through the adsorbed protein to the surface of the adsorbate.

Auger electron spectroscopy does not distinguish small changes in electron binding energy as does XPS and it is possible that those investigators were confusing sulfur from cysteine and cystine residues in plasma proteins for heparin sulfur. The XPS results report in this dissertation clearly demonstrate that the immobilized heparin was not able to penetrate adsorbed proteins and become associated with the surface of the protein layer, casting doubts on the hypothesis of Larsson (81) that the anti-coagulant effect of immobilized heparin is due to its ability to migrate to the surface of the adsorbed protein layer and then interact with free coagulation factors. The fact that immobilized heparin is masked by adsorbed proteins supports the contention that prolonged in vitro clotting times observed with immobilized heparin surfaces are the result of plasma depletion of coagulation factors. The observation that the 12 carbon spacer arm immobilized heparin after plasma contact was lower in sulfur at 227 eV, 0.1 atomic percent, than the 4 carbon spacer arm immobilized heparin, 0.2 atomic percent sulfur, would indicate that the protein layer adsorbed onto the 12 carbon spacer arm immobilized heparin contained more ATIII than did the 4 carbon spacer arm immobilized heparin; no sulfur was detected near 227 eV with adsorbed ATIII. This possibility would support the APTT test results where it was hypothesized that heparin immobilized with the

longer spacer arms was preferentially interacting with ATIII and subsequent coagulation factors more specifically than heparin immobilized with shorter spacer arms.

4.3 Platelet Retention and PF4 Release

Platelet interactions with a heparinized surface can play a major role in the in vivo thromboresistance of that surface. Soluble heparin has been shown to adversely interact with platelets, resulting in aggregation and the release of endogenous platelet substances which can activate and accelerate thrombotic mechanisms. Platelet factor 4 is such a substance. Released from α-granules within the platelets, PF4 binds to and neutralizes the anticoagulant activity of heparin; therefore, were platelets activated by immobilized heparin surfaces, PF4 could be released and bind to the immobilized heparin rendering the surface ineffective with regards to heparin interactions with coagulation factors.

In these experiments, platelet retention and PF4 release were evaluated simultaneously using column techniques where whole blood was perfused through columns containing heparin immobilized beads. With this type of experimental setup, platelet retention values reflect both platelet adhesion onto the test materials and platelet aggregation in the plasma phase induced by the test materials, where platelet aggregates are retained based on size in the interstitial spacings between packed beads. In addition to providing information on the degree of PF4 binding to and neu-

tralization of immobilized heparin, simultaneously conducting platelet retention and PF4 release experiments provides a more thorough characterization of platelet thrombotic effects associated with platelet/material interactions by demonstrating both platelet retention and platelet activation as indicated by PF4 release. Platelet retention does not necessarily imply resulting thrombosis.

Platelet factor 4 levels in whole blood exposed to heparinized surfaces, and control surfaces, were therefore monitored to provide an indication of platelet activation and degranulation due to contact with various surfaces and to determine the amount of released PF4 bound to immobilized heparin surfaces. As shown in Figure 33, platelet retention for heparin immobilized to 4 carbon and 12 carbon diaminoalkane derivatized Sepharose 6-MB beads is statistically indistinguishable; these values, for comparable fraction numbers, being also indistinguishable from untreated Sepharose 6-MB beads. The same response was observed for diaminobutane derivatized Sepharose 6-MB without immobilized heparin; however, the diaminododecane derivatized beads produced significant platelet retention. The same trends were observed for PF4 release, as shown in Figure 34. Control Sepharose 6-MB elicited the least PF4 release while both heparinized materials were indistinguishable, with regards to PF4 release, for comparable fraction numbers. Once again, the diaminododecane derivatized gels produced high levels of PF4 release, at least three times higher than all other materials evaluated.

The correlation between platelet retention and PF4 release provides documentation of the high thrombogenicity of the diaminododecane derivatized surfaces. The increased hydrophobic nature of the diaminododecane derivatized gels is the only parameter that differs between it and the diaminobutane derivatized gels, both surfaces having the same molar degree of diaminoalkane derivatization. These studies show that platelets are both retained and activated by diaminododecane surfaces. Although spacer arm lengths affect in vitro clotting times for immobilized heparin materials, platelet interactions were indistinguishable for the two spacer arms evaluated. This is not a totally unexpected result. As previously stated, the immobilized heparin was shown to be completely covered by the adsorbed protein layer; platelets do not "see" immobilized heparin. Rather, the nature of the adsorbed protein surface on top of the immobilized heparin controls platelet interactions. For this reason, immobilized heparin might be more benign with regards to platelet interactions than soluble heparin.

It is interesting to note that more PF4 was eluted off heparin immobilized to the 12 carbon spacer arm than the 4 carbon spacer arm. With a molecular weight of $\sim$ 27,000 (101), the 12 carbon spacer arm heparin bound $\sim 2.3 \times 10^{-11}$ moles of PF4, while the 4 carbon spacer arm bound $\sim 1.4 \times 10^{-11}$ moles of PF4. Assuming a molecular weight of 10^4 for the immobilized heparin, the 12 carbon and 4 carbon spacer arm materials both had $\sim 1.5 \times 10^{-7}$ moles of immobilized heparin; therefore, much less than 1% of the

immobilized heparin was neutralized by PF4 released during the whole blood perfusion experiments.

4.4 Summary Conclusion

From these studies, the following conclusions are made:

1. Primary amine groups on heparin can be blocked with no resultant loss in anticoagulant potency. Carboxylic and hydroxyl groups on heparin can be partially blocked with minimal resultant losses in anticoagulant activity; however, the degree of carboxylic blocking is critical and more than approximately 50% blockage results in the total loss of anticoagulant activity. The end groups on ligands used to derivatize the carboxylic and hydroxyl groups appear to have little affect on the anticoagulant activity of partially derivatized heparins. At approximately 10 to 20% blockage of both carboxylic and hydroxyl groups; methyl, carboxylic and sulfate end groups on derivatized ligands all produce derivatized heparins with approximately 120 units/mg. The initial activity of the starting material heparin was approximately 155 units/mg.

2. Based on prior n-butylamine blocking reactions of heparin carboxylic groups, heparin was immobilized via analogous reaction schemes to diaminoalkane derivatized polymer substrates. The n-butylamine heparin amidation reactions showed that 20% blockage of the total heparin carboxylic groups resulted in active heparin. The n-butylamine was used to mimic the diaminoalkane spacer arms used in the immobilization reactions where a

maximum of 20% of the total heparin carboxylic groups participated in the covalent surface immobilization, assuming 100% efficiency for the immobilization reactions. The fact that 20% blockage of heparin carboxylic groups resulted in active heparin indicated that the immobilized heparin, also utilizing 20% of the total carboxylic groups, would be active.

3. The heparin immobilization yields were found to be dependent upon the diaminoalkane spacer arm length. Immobilization yields progressively increase with 2, 4 and 8 carbon spacer arms, the diaminooctane spacer producing the maximum immobilization yield. The immobilization yields then progressively decrease with 10 and 12 carbon diaminoalkane spacer arms.

4. It was found that APTT clotting times of plasma exposed to immobilized heparin materials were also dependent upon the length of the diaminoalkane spacer arm. Elevations in APTT, relative to control plasma, were observed with 2, 4 and 8 carbon spacer arms; however, beginning with 10 carbon spacer arms, APTT dramatically increased, presumably due to more specific binding interactions with ATIII and subsequent coagulation factors.

5. XPS analyses of immobilized heparin materials, with 4 carbon and 12 carbon diaminoalkane spacer arms, before and after contact with single component solutions of ATIII, albumin and fibrinogen and with plasma revealed that heparin is covered by absorbed proteins and is not associated with the surface of the protein adsorbate. The nature of the adsorbed proteins, from contact with plasma, appears different for heparin immobilized by

4 carbon and 12 carbon diaminoalkane spacer arms. The 4 carbon spacer arm immobilized heparin produces a protein adsorbate higher in sulfur than the 12 carbon spacer arm immobilized heparin. Since it was demonstrated that adsorbed ATIII surfaces were essentially free of sulfur, this might indicate that the 12 carbon spacer arm immobilized heparin is preferentially interacted with ATIII.

6. Platelet retention and PF4 release were indistinguishable for heparin immobilized with 4 carbon and 12 carbon spacer arms and these responses were comparable to untreated Sepharose beads. The diaminododecane derivatized Sepharose produced high levels of platelet retention and PF4 release, these affects on platelet function being eliminated following heparin immobilization. Since it was previously shown that heparin immobilized with 4 and 12 carbon spacer arms was covered by adsorbed proteins and therefore not able to directly interact with platelets, the fact that platelets did not adversely interact with immobilized heparin surfaces, as was feared based on the platelet behavior of soluble heparin, is understandable.

7. Based on the amount of PF4 eluted off immobilized heparin materials by 1 M NaCl, less than 1% of the immobilized heparin was neutralized by PF4. Since relatively minor amounts of PF4 were released from platelets in whole blood following contact with immobilized heparin materials, this too is not totally unexpected.

8. Anticoagulant activity of immobilized heparin is depen-

dent upon the spacer arm length while platelet interaction is independent of the spacer arm length.

4.5 Proposed Future Studies

4.5.1 Immobilization of Heparin Fractions

All of the work reported in this thesis was conducted on unfractionated, bulk heparin. Numberous investigators have studied molecular weight fractions of heparin, including high ATIII affinity molecular weight fractions. In general, low molecular weight fractions have less anticoagulant activity but improved platelet interactions than do the high molecular weight fractions. An exception to this rule of thumb is the low molecular, high ATIII affinity heparin fractions which possess high antifactor Xa activity. Since platelet interactions with immobilized bulk heparin, ranging in molecular weight from less than 10,000 to greater than 20,000, have been demonstrated to be relatively benigh, presumably due to the fact that the immobilized heparin was covered with adsorbed proteins that did not adversely interact with platelets, significant improvements in platelet interactions might not be achieved by immobilizing low molecular weight heparin fractions. However, improved intrinsic coagulation factor interactions might be achieved through the immobilization of high ATIII affinity fractions. It is therefore proposed that such high ATIII affinity fractions be isolated and immobilized for further thromboresistance characterizations using

both high (> 20,000) and low (< 10,000) molecular weight high affinity fractions.

4.5.2 Protein Adsorption Isotherms

The nature of the proteins adsorbed onto the heparinized materials is of great importance in the thromboresistance of such surfaces. Increased anticoagulant activity of immobilized heparin with increased spacer arm length was observed. It was hypothesized that this effect was due to increased ATIII interactions with the longer spacer arms, yet this hypothesis was not proven. It is therefore proposed that competitive adsorption isotherms be conducted with purified radiolabeled ATIII, albumin and fibrinogen solutions (three separate solutions where only one protein is labeled) for heparin immobilized via diaminobutane and diaminododecane spacer arms. It would also be interesting to conduct platelet adhesion studies on adsorbed ATIII surfaces. Benign platelet interaction has typically been attributed to albumin adsorption; however, platelet interaction with adsorbed ATIII surfaces has not been investigated. At a plasma concentration near 0.2 μg/ml ATIII is a relatively abundant plasma protein that could also be adsorbing onto materials to produce benign surfaces with regards to platelet interaction.

4.5.3 In Vivo Characterization

Finally, both acute and chronic *in vivo* thromboresistance characterization studies should be conducted. Parameters that

should be investigated during this phase of experimentation should include, the *in vivo* stability of the immobilized heparin (the heparin is coupled by a degradable amide bond) and evaluations of surface thrombosis on heparinized materials following venous implantation.

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